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ARTICLE

DNA methylation of *TOMM40-APOE-APOC2* in Alzheimer's disease

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Abstract

The apolipoprotein E (*APOE*) ε4 allele is the major genetic risk factor for Alzheimer's disease (AD). Multiple regulatory elements, spanning the extended *TOMM40-APOE-APOC2* region, regulate gene expression at this locus. Regulatory element DNA methylation changes occur under different environmental conditions, such as disease. Our group and others have described an *APOE* CpG island as hypomethylated in AD, compared to cognitively normal controls. However, little is known about methylation of the larger *TOMM40-APOE-APOC2* region. The hypothesis of this investigation was that regulatory element methylation levels of the larger *TOMM40-APOE-APOC2* region are associated with AD. The aim was to determine whether DNA methylation of the *TOMM40-APOE-APOC2* region differs in AD compared to cognitively normal controls in post-mortem brain and peripheral blood. DNA was extracted from human brain ($n = 12$) and peripheral blood ($n = 67$). A methylation array was used for this analysis. Percent methylation within the *TOMM40-APOE-APOC2* region was evaluated for differences according to tissue type, disease state, AD-related biomarkers, and gene expression. Results from this exploratory analysis suggest that regulatory element methylation levels within the larger *TOMM40-APOE-APOC2* gene region correlate with AD-related biomarkers and *TOMM40* or *APOE* gene expression in AD.

Introduction

The apolipoprotein E (*APOE*) ε4 genetic variant is the strongest genetic risk factor for late-onset Alzheimer's disease (AD) described to date. Fine mapping of the *APOE* locus genetic architecture, including the promoter and regulatory regions across an extended *APOE* locus cluster of genes (*TOMM40*, *APOE*, *APOC1*, *APOC4*, *APOC2*), in both AD and early stage AD (mild cognitive impairment (MCI)), implicates strong disequilibrium with the *APOE* ε4 allele [1–4]. A complex regulatory structure has been described at this extended region that includes multiple enhancers [5–13] suggesting that multiple regulatory elements contribute to apoE levels, as well as other genes, across a 64,000 base pair genomic region (Fig. 1).

Interestingly, in humans and in mouse models, *APOE* ε2 carriers have higher apoE levels, compared to ε3 and ε4 (ε2 > ε3 > ε4) and are protected against AD pathology, including the accumulation of toxic Aβ protein (ε2 < ε3 < ε4) [4, 14–16]. ApoE4 is less efficient in transporting lipids [17–21] and is associated with a detrimentally decreased clearance and increased deposition of Aβ peptides in AD brain, compared to apoE2 or apoE3 [22, 23]. ApoE levels are, by most accounts, low in AD [4, 14–16] suggesting that an increase in apoE, albeit without an increase of detrimental apoE4, may be beneficial in AD. Indeed, a recent clinical trial tested the RXR-selective retinoid agonist, bexarotene, as a means to enhance *APOE* and *ABCA1* promoter activity with the goal of inducing apoE lipidation and enhancing the removal of Aβ from the brain in AD patients [24]. The primary outcome of this clinical trial was negative but suggested that bexarotene reduced brain Aβ and increased serum Aβ in ApoE4 non-carriers [24]. This clinical trial, and other research focusing on modulation of

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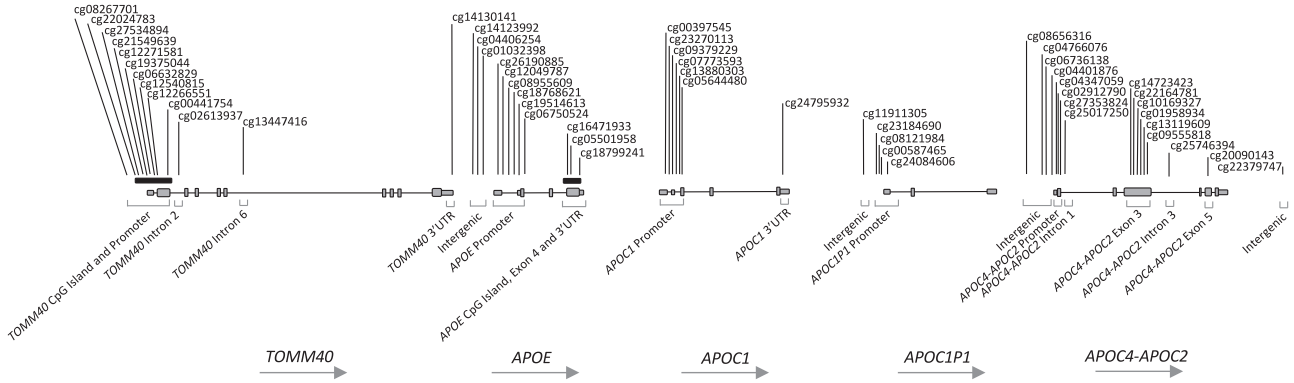

Fig. 1 Genomic map of *APOE* locus CpG sites. All genes are transcribed in the same direction as shown here from left to right. CpG islands are black bars. Gene exons are gray bars. Approximate CpG locations and cg ID are noted as cg number from the Infinium HumanMethylation450 BeadChip Kit (Illumina). Genes are located at chr19:45,392,813–45,456,635 UCSC Genome Browser Human Feb. 2009 (GRCh37/hg19) Assembly. Approximate 64,000 base pair region is not to scale

apoE, emphasizes the need to fully understand the regulatory mechanisms underlying *APOE* gene regulation.

Regulatory element activity, such as promoter and enhancer activity can be influenced by cytosine methylation at CpG sites in the genome [25]. Hypermethylated promoters are largely associated with gene expression inhibition and hypermethylated non-promoter regions located within enhancer regions have been associated with loss of enhancer activity and transcriptional inactivation of target genes [26]. However, in some circumstances, hypermethylation has been associated with enhanced expression of some genes. For example, the AD-related gene, *TREM2*, has been reported to have a hypermethylated promoter that is associated with enhanced *TREM2* expression [27].

DNA methylation has been described as significantly associated with increasing age and age-related diseases in both human tissue [28–34] and mouse models [35, 36]. Changes in DNA methylation in AD patients and AD mouse models suggests that DNA methylation may be associated with AD pathology [35, 37, 38]. Furthermore, because most AD cases have a clinical onset over the age of 65 years and AD is strongly associated with age, it has been suggested that methylation may play a critical role in AD risk [28–34].

Methylation status of the *APOE* gene has been described [39–43]. The *APOE* gene has a bimodal methylation structure, with a hypomethylated CpG poor promoter and a comparatively hypermethylated CpG-island located in the *APOE* exon 4 to 3′ UTR region [39–43]. Methylation of the surrounding genomic regulatory regions, such as promoters and 3′ UTRs, are less well studied.

Given that there is an extended region of regulatory elements that span across the extended *APOE* locus (*TOMM40-APOE-APOC2)* [5–13], the aim of this investigation was to describe the DNA methylation status of the entire extended locus and the relationship with tissue type, disease status, gene expression, or AD-related biomarkers in post-mortem brain or peripheral blood. Therefore, a biased exploratory study using whole-genome methylation data from post-mortem brain and whole blood was performed that focused only on an extended region surrounding *APOE* where multiple regulatory elements have been described. Results show that methylation of the extended *APOE* locus is different between brain and blood, and is associated with disease, gene expression, and AD-related biomarkers.

Materials and methods

DNA samples

Post-mortem brain samples were obtained from the University of Washington Alzheimer's Disease Research Center (Table 1). DNA from post-mortem brain was extracted using the Qiagen Allprep DNA/RNA Mini Kit (Qiagen) according to the manufacturer instructions. Blood samples were obtained from the Cleveland Clinic Center for Brain Health Biobank (CBH Biobank). All samples were obtained from subjects who had consented to donate biospecimens to the CBH Biobank. All CBH Biobank subjects met their respective disease diagnostic guidelines [44–49] for MCI, AD, or cognitively normal controls following a consensus conference that included two behavioral neurologists. Cognitively normal controls age- and sex-matched to MCI and AD subjects in the CBH Biobank were included (Table 1). Blood was collected during life and DNA was extracted from the all cell pellet using the QIAamp Blood Maxi kit (Qiagen).

A replication cohort was included for validation (Table 1) [50]. The replication cohort data were obtained from Gene Expression Omnibus (GEO) (Accession GSE59685) with permission. This study from GEO used the

Table 1 DNA sample description

	Controls		AD	*p*-Value
(A) Post-mortem brain				
n	6		6	
% Female	50		50	
% *APOE* ε4+	50		50	
Age mean (Std. Dev.)	88 (5.7)		79 (10.9)	0.014
Braak stage	II–IV		IV–VI	<0.001
Neuritic plaque score	Absent-moderate		Sparse-frequent	0.003
	Controls	MCI	AD	*p*-Value
(B) Whole blood				
n	24	17	26	
% Female	58	47	54	
% *APOE* ε4+	42	59	69	
Age mean (SD)	66 (4.1)	64 (9.3)	65 (8.1)	
Biomarker *n*	5	17	24	
CSF $A\beta_{42}$ mean pg/ml (SD)	1293 (730)	502 (374)	425 (174)	0.003[a]; <0.001[b]
CSF T-Tau mean pg/ml (SD)	567 (520)	532 (342)	671 (348)	
CSF P-Tau mean pg/ml (SD)	66 (31)	74 (51)	113 (50)	0.042[b]; 0.010[c]
	Controls		AD	
(C) Replication cohort				
Post-mortem brain				
n	23		60	
% Female	43		65	
Age mean (SD)	76 (13.3)		86 (7.3)	
Whole blood				
n	9		48	
% Female	67		71	
Age mean (SD)	80 (5.8)		83 (6.9)	

DNA was collected from brain obtained from the University of Washington: Alzheimer's Disease Research Center Brainbank (UW-ADRC Brainbank) (A). Whole-blood DNA from the Cleveland Clinic Lou Ruvo Center for Brain Health: Aging and Neurodegenerative Disease Biobank (CBH-biobank) (B). Replication cohort data from Gene Expression Omnibus (GEO) (Accession GSE59685) (C). Only significant *p*-values are shown

[a] Significant difference between Controls and MCI

[b] Significant difference between Controls and AD

[c] Significant difference between MCI and AD

same methylation analysis method as described here from human cerebellum and peripheral blood from AD and controls. Percent methylation beta values from this previously published cohort from London were pulled from GEO and analyzed [50].

All sample collection and consent was approved by the respective institutional review boards.

Methylation analysis

Genomic DNA (500 ng) was bisulfite converted using the EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA) and hybridized on Infinium HumanMethylation450 BeadChip Kit (Illumina) according to the manufacturer's protocols (Illumina). Signal intensities were measured using an Illumina iScan BeadChip scanner. Sample identity of DNA methylation was confirmed with genotype data using MixupMapper [51]. SNPs available on the platform were used to confirm the genotype data using RnBeads [52]. Quality control (QC) on the DNA methylation data was performed using the R package MethylAid [53]. Ambiguously mapped probes with a low bead count (<3 beads), and probes with a low success rate (missing in >95% of the samples) were not included in further analyses and included extended *APOE* locus CpGs: cg21879725, cg13496662, cg11337525, cg17769836, and cg27436184. DNA methylation values are the percent methylated (beta-values) at any given CpG site (cg) for each DNA sample within the extended *APOE* locus using Genome Studio (Methylation module v1.8). The extended *APOE* locus was defined as chr19:45,392,813–45,456,635 using UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly (Fig. 1). Beta values for cgs located in the extended *APOE* locus were exported from GenomeStudio (Methylation module v1.8).

Quantitative traits

RNA was extracted from brain samples using the Allprep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Expression of *TOMM40* or *APOE* and *ACTB* were measured in the hippocampus using qRT-PCR and are presented as relative qRT-PCR ($\Delta CT = TOMM40$ *or APOE—Actin*). Cerebrospinal fluid AD-related biomarkers were measured using a Millipore $A\beta_{42}$, total-tau (T-tau) and phosphorylated-tau 181 (P-tau) kit (Millipore) and a Luminex xMap 200 system (Luminex).

Statistical analysis

Candidate CpG were additionally filtered as follows. First, probe sequences were aligned to the reference human genome using Bowtie 2 [54] to assess the potential to cross-hybridize to multiple genomic locations, thus affecting DNA methylation measurements [55]. CpG loci targeted by cross-hybridizing probes (defined as those lacking unique genome alignments, with up to three base mismatches) were excluded from further consideration. Second, potential

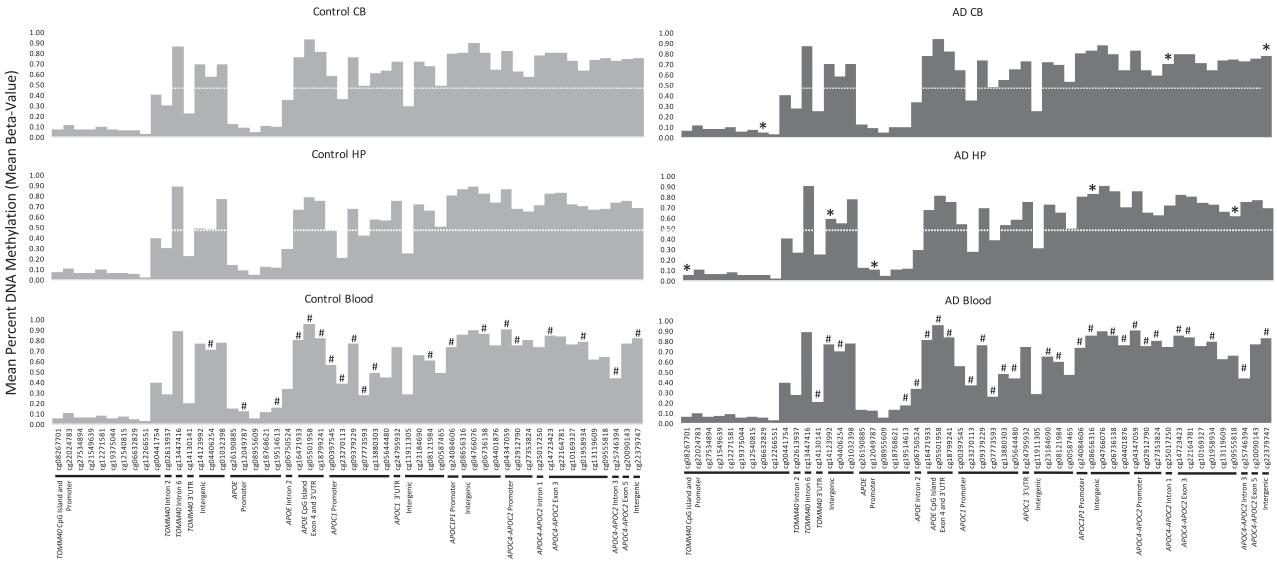

Fig. 2 Percent DNA methylation averages (means) across *APOE* locus. Control cerebellum (CB: $n = 6$), AD CB ($n = 6$), control hippocampus (HP; $n = 6$), AD HP ($n = 6$), control peripheral blood (PB: $n = 24$), and AD PB ($n = 26$) mean beta-values (percent DNA methylation) vary by genomic region, tissue type, and disease status. White dotted line is set to beta-value 0.50 for reference. Asterisk (*) denotes significantly different CpG mean for AD compared to controls ($p < 0.05$) using a multivariate analysis where CpG is the dependent variable and the independent variable (fixed factor) is disease status or tissue type. There was not a significant difference between control and AD in PB. The pound sign (#) represents a significant difference between control HP compared to control PB or AD HP compared to AD PB

sources of genetic confounding and context disruption for DNA methylation (such as polymorphisms at the CpG locus) were identified by retrieving known genetic variations and computing the corresponding minor allele frequencies (MAFs) in the European population, based on publicly available data generated by the 1000 Genomes project. As a precautionary measure, CpG loci found within 100 base pairs (bp) of non-rare variants (minor allele frequency <1%) were removed from the list of candidates. CpG with missing variables (failed CpG) were eliminated from the analysis and included extended *APOE* locus CpGs: cg21879725, cg13496662, cg11337525, cg17769836, and cg27436184. Correlations between either mRNA expression or AD-related biomarkers were tested using linear regression where percent methylation was the dependent variable and mRNA or AD-related biomarker was the independent variable within each disease group. Multivariate analyses or linear regression were performed for all analyses using SPSS (SPSS Version 22). Given that this analysis included 54 CpG out of 450,000 CpG available on the Infinium HumanMethylation450 BeadChip Kit (Illumina) no significance was found if multiple comparisons for all CpG available on this platform were taking into account.

There is a complex regulatory structure at this extended *APOE* locus that may include competition for scarce transacting resources, such as methylation, between genes. To address whether the methylation of one gene might be necessary to allow for full expression of another, a linear regression analysis was performed to test whether gene expression of one gene (i.e. *TOMM40*) is negatively correlated with methylation while the other gene (i.e. *APOE*) is positively correlated in the brain. Therefore, CpG beta value was the dependent variable and gene expression was the independent variable in the linear regression models both with and without both genes (Fig. 3, Supplementary Fig. 2; Supplementary Tables 3 and 4). In addition, to test whether pathological conditions in the brain influence methylation status at the *APOE* locus, CpG beta value was the dependent variable and CSF biomarker was the independent variable in some linear regression analyses. Multiple comparison corrections were performed using the Holm multiple comparison method [56]. The 54 CpG sites tested were designated as the number of multiple comparisons ($n = 54$). The Holm adjusted p-values for the analyses are shown in the Supplementary Tables [56]. None of the comparisons are significant if 54 multiple comparisons are considered. Significance was set at a p-value of <0.050 for any given CpG methylation beta-value analyzed and these p-values are shown in the figures as well as the Supplementary Tables.

Results

Tissue-dependent methylation at the extended *APOE* locus

AD and cognitively normal control percent methylation (beta-value) for each CpG across the extended *APOE* locus (Fig. 1) from brain hippocampus (HP) DNA, cerebellum (CB) DNA, and peripheral blood (PB) DNA were compared (Fig. 2; Table 1: panels A and B; Supplementary Table 1). Given that cerebellum (CB) is a less affected region in AD, compared to HP, it was also analyzed to demonstrate differences in DNA methylation between CB and HP as well as PB (Fig. 2; Table 1). Two hypomethylated regions in all three tissues were identified, one located at the *TOMM40* promoter and the other located at the *APOE* promoter (Fig. 2). In addition, CpG across the locus were significantly different between AD and cognitively normal controls in the CB and HP, but no CpG were significantly different between AD and cognitively normal controls in PB (Fig. 2). Notably, there were differences between tissues, where cognitively normal control HP methylation were significantly different compared to cognitively normal control PB, and AD HP methylation were significantly different compared to AD PB. Interestingly, methylation of *TOMM40* promoter CpG were not significantly different between HP and PB (Fig. 2). These results suggest that the extended *APOE* genomic region is methylated differently in blood compared to brain in most regions, but not in the *TOMM40* promoter region. Furthermore, significant methylation differences between AD and controls were identified in HP and CB, but not PB.

Hippocampus and cerebellum methylation

HP or CB percent methylation for each CpG across the extended *APOE* locus was compared between AD and cognitively normal controls using a multivariate analysis where all CpG beta-values were the dependent variables and AD compared to cognitively normal controls was the independent variable (Supplementary Fig. 1A). In the HP, five CpG were significantly different between AD and controls ($p < 0.050$); *TOMM40* promoter (cg08267701), *TOMM40-APOE* intergenic region (cg14123992), *APOE* promoter (cg12049787), *APOC1P1-APOC4* intergenic region (cg08656316), and *APOC4-APOC2* exon 3 (cg09555818) (Fig. 2; Supplementary Fig. 1A; Supplementary Table 2). In the CB, three CpG were significantly different between AD and controls ($p < 0.050$); *TOMM40* promoter (cg06632829), *APOC4-APOC2* promoter (cg25017250) and Intergenic (cg22329747) (Fig. 2; Supplementary Fig. 1B; Supplementary Table 2). A replication cohort was used to validate these results. The replication cohort data were obtained from GEO and consists of DNA methylation data from CB. Nine CpG showed a significant difference in the replication cohort (Supplementary Fig. 1C; Supplementary Table 7). The *TOMM40* promoter region replicated a significant difference in methylation between AD and control CB. These results suggest that the extended *APOE* genomic region is methylated differently in AD compared to controls in the HP and CB.

The relationship between *APOE* locus CpG methylation and *TOMM40 or APOE* mRNA expression in our HP samples was evaluated using linear regression analyses. *APOE* expression significantly correlated with *TOMM40* promoter cg22024783 in the group as a whole (All; Fig. 3A), *TOMM40* intron 6 cg13447416 in controls, *APOE* promoter cg26190885 within AD, *APOE* promoter cg08955609 within controls and *APOE* CpG island cg16471933 within all and controls (Supplementary Fig. 2A; Supplementary Table 3). *TOMM40* expression significantly correlated with *TOMM40* promoter cg06632829 in AD, *TOMM40* promoter cg1266551 in controls, *TOMM40* Intron 6 cg13447416 with AD, *APOE* CpG island cg18799241 within the groups as a whole (All), *APOC1* promoter cg23270113 in AD, cg09379229 in All, cg13880303 in AD, *APOC1P1* promoter cg24084606 in AD, *APOC4-APOC2* cg04347059 in All, *APOC4-APOC2* exon 3 cg14723423 and cg13119609 in AD (Supplementary Fig. 2B; Supplementary Table 3). Interestingly, CpG within the *TOMM40* promoter showed both a significant association with AD (cg08267701: Supplementary Fig. 2A) and a correlation with both *APOE* (cg22024783) and *TOMM40* (cg06632829, cg12266551) expression (Supplementary Fig. 2A, B). CpG within the *APOE* promoter (cg12049787) were associated with AD (Supplementary Fig. 1A) and correlated with *APOE* expression (cg26190885, cg08955609) (Supplementary Fig. 2A). CpG within *APOC4-APOC2* exon 3 (cg09555818) were associated with AD (Supplementary Fig. 1A) and correlated with *TOMM40* expression in the entire group (All: cg14723423) or in AD (cg13119609, cg09555818) (Supplementary Fig. 2B; Supplementary Table 3). Taken together, these results suggest that there is an association between methylation and gene expression at this locus that might be related to disease status.

TOMM40 promoter methylation was associated with disease (cg08267701: Supplementary Fig. 1A) as well as both *APOE* (Fig. 3A; cg22024783) and *TOMM40* (cg06632829, Fig. 3A cg12266551) expression (Supplementary Fig. 2A, B), and methylation of two of these CpG showed opposing correlation (Fig. 3A; Supplementary Table 4). The *TOMM40* promoter cg22024783 showed a negative non-significant correlation with *TOMM40* expression and a significant positive correlation with *APOE* expression (Fig. 3A), suggesting that a decrease in

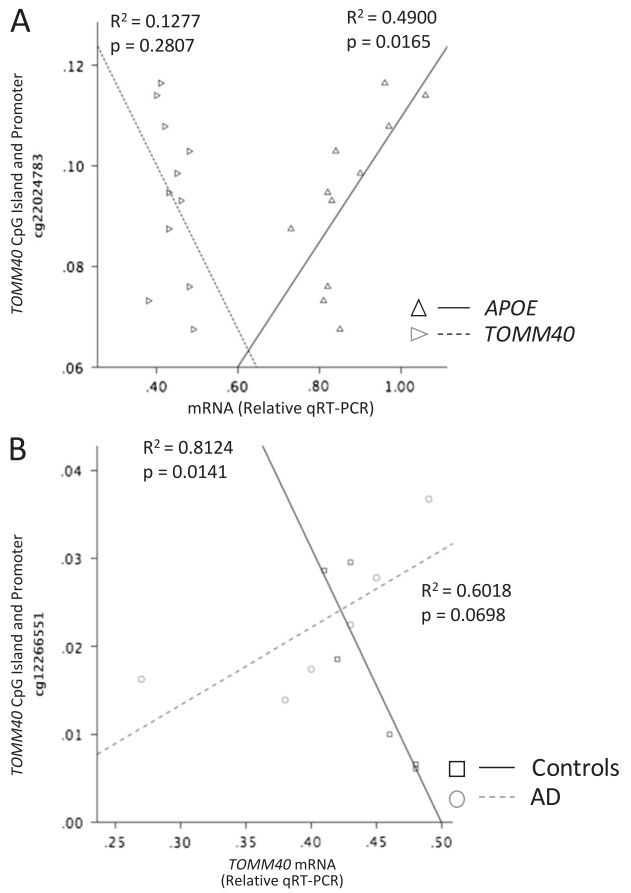

Fig. 3 *TOMM40* promoter CpG methylation is correlated with RNA expression *TOMM40* promoter cg22024783 methylation in all subjects (AD and controls) is significantly positively correlated with *APOE* mRNA expression and non-significantly negatively correlated with *TOMM40* mRNA expression in HP (**a**). *TOMM40* promoter cg12266551 methylation is significantly negatively correlated with *TOMM40* mRNA expression in controls and non-significantly positively correlated with *TOMM40* mRNA expression in AD in HP (**b**)

methylation of the *TOMM40* promoter cg22024783 may be related to an increase in *TOMM40* expression and conversely a decrease in *APOE* expression. However, the corresponding negative correlation with *TOMM40* expression for *TOMM40* promoter cg22024783 was not significant (Fig. 3A and was not associated with AD (Supplementary Fig. 1A; Supplementary Table 2) . Another *TOMM40* promoter CpG (cg12266551) did show a difference between AD and controls where a negative correlation in control *TOMM40* expression was in the opposite direction in AD (marginally positively correlated) (Fig. 3B). These results suggest that *TOMM40* methylation may influence both *TOMM40* and *APOE* expression and may be disrupted in AD compared to controls.

Peripheral blood DNA methylation

Percent methylation for each CpG across the extended *APOE* locus were compared between cognitively normal controls (Controls), mild cognitive impairment (MCI), AD, and MCI, AD (Supplementary Fig. 3A; Supplementary Table). All comparisons were performed using a multivariate analysis where all CpG beta-values were the dependent variables and group comparison (Controls vs. MCI, Controls vs. AD, Controls vs. MCI and AD, MCI vs. AD) was the independent variable (Supplementary Fig. 3A; Supplementary Table 5). Methylation of multiple CpG were significantly different between groups, including the following: *TOMM40* promoter (Controls vs. MCI or Controls vs. MCI and AD: cg22024783; MCI vs. AD: cg12271581, cg0663829), *APOE* promoter (Controls vs. AD: cg26190885; Controls vs. MCI and MCI vs. AD; cg120449787; MCI vs. AD; cg19514613), *APOE* CpG island (Controls vs. MCI or Controls vs. MCI and AD: cg05501958, cg18799241) and the *APOC1* promoter (Controls vs. MCI: cg23270113, cg13880303), *APOC1P1-APOC4-APOC2* intergenic (cg08656316) (Supplementary Fig. 3A). DNA methylation in PB for *APOE* promoter, the *APOE* CpG island and the *APOC1* promoter was significantly different between disease groups for our cohort and for the replication cohort (Supplementary Fig. 3A, B; Supplementary Table 7). However, there was a lack of a significant difference between AD and controls in our cohort, while there was a significant difference between AD and controls in the replication cohort for several regions across this extended locus (Supplementary Fig. 3B; Supplementary Table 7). These results suggest that the extended *APOE* genomic region is methylated in the blood differentially according to disease status.

To determine if an association between *APOE* locus methylation was related to specific underlying pathology, CSF $A\beta_{42}$, total-tau (T-tau) and phosphorylated-tau_{181} (P-tau) levels, were correlated with PB methylation in each group (Controls, MCI, AD) (Supplementary Fig. 4A–C; Supplementary Table 6). CSF $A\beta_{42}$ levels significantly ($p < 0.050$) correlated with *TOMM40* promoter CpG (AD: cg22024783; MCI cg19375044), *TOMM40* intron 2 (AD: cg02613937), *TOMM40* intron 6 (MCI: cg13447416), *TOMM40-APOE* intergenic (MCI: cg14123992), *APOC1* promoter (AD: cg00397545; Controls: cg09379229; AD: cg05644480), *APOC1-APOC1P1* intergenic (MCI: cg08121984), *APOC4-APOC2* promoter (controls: cg27353824) (Supplementary Fig4A). CSF T-tau levels significantly ($p < 0.05$) correlated with *TOMM40* promoter CpG (Controls: cg22024783), *TOMM40-APOE* intergenic (AD: cg04406254), *APOE* promoter (MCI: cg18768621), *APOE* CpG (AD: cg05501958), *APOC1* promoter (controls: cg09379229), *APOC1P1-APOC4-APOC2* intergenic

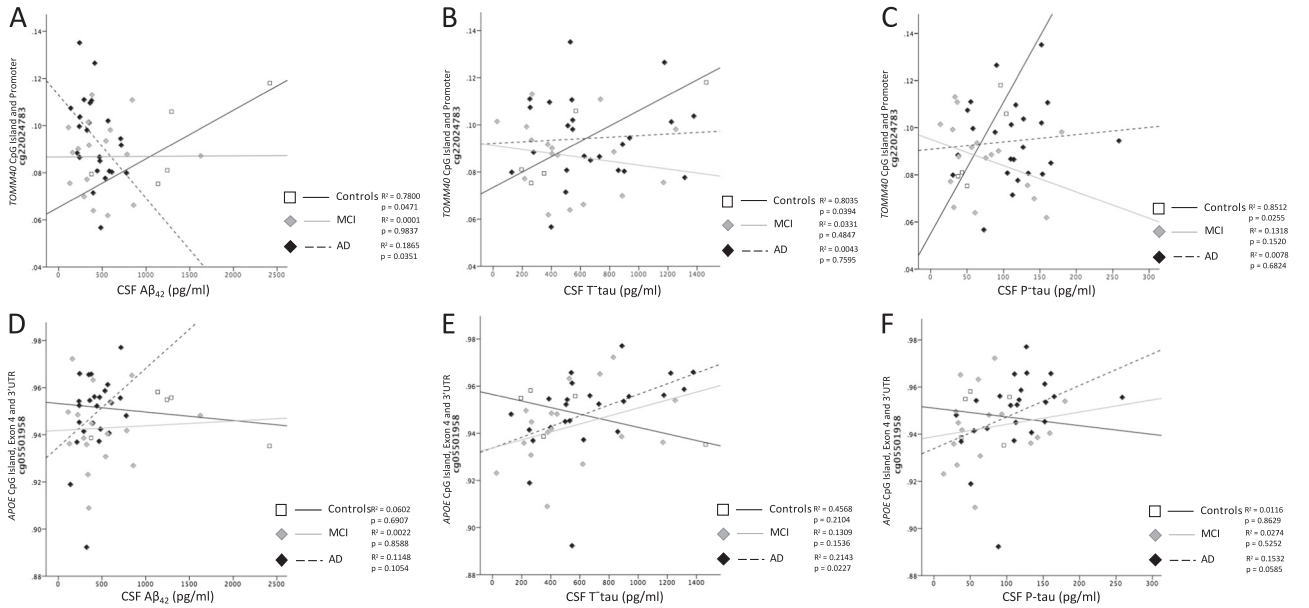

Fig. 4 *APOE* locus CpG methylation is correlated with AD-related biomarkers. *TOMM40* promoter cg22024783 methylation is significantly positively correlated with CSF $A\beta_{42}$ in controls, not correlated in MCI, and significantly negatively correlated in AD (**a**). *TOMM40* promoter cg22024783 methylation is significantly positively correlated with CSF T-tau in controls, not in MCI or AD (**b**). *TOMM40* promoter cg22024783 methylation is significantly positively correlated with CSF P-tau in controls, in MCI or AD (**c**). *APOE* CpG island cg05501958 methylation is not significantly correlated with CSF $A\beta_{42}$ in controls, MCI or AD (**d**). *APOE* CpG island cg05501958 methylation not correlated with CSF T-tau in controls or MCI, but is significantly positively correlated in AD (**e**). *APOE* CpG island cg05501958 methylation is not correlated CSF P-tau in controls or MCI, but is marginally positively correlated with CSF P-tau in AD (**f**)

(AD: cg04766076), *APOC4-APOC2* promoter (Controls and AD: cg04347059; Controls cg27353824), *APOC4-APOC2* exon 3 (AD: cg14723423, cg10169327), *APOC4-APOC2* exon 5 (MCI: cg20090143) (Supplementary Fig. 4B). CSF P-tau levels significantly ($p < 0.050$) correlated with *TOMM40* promoter CpG (Controls: cg22024783), *TOMM40-APOE* intergenic (controls: cg01032398), *APOE* promoter (MCI: cg18768621; AD: cg19514613), *APOE* CpG (MCI: cg16471933), *APOC1* promoter (AD: cg05644480), *APOC1P1-APOC4-APOC2* intergenic (AD: cg04766076), *APOC4-APOC2* exon 3 (MCI: cg22164781), *APOC4-APOC2* exon 5 (MCI: cg20090143) (Supplementary Fig. 4C). Since *TOMM40* CpG island and the *APOE* CpG island methylation is associated with differences between disease groups (Supplementary Fig. 1A) as well as AD-related biomarkers (Supplementary Fig. 4A–C) these results implicate a relationship between underlying AD-related pathology and methylation at this locus.

Next, we evaluated whether methylation of CpG in this region were positively or negatively correlated (Fig. 4). Control, but not MCI, CSF $A\beta_{42}$, levels were significantly positively correlated with the *TOMM40* promoter cg22024783 while AD CSF $A\beta_{42}$, levels were significantly negatively correlated (Fig. 4a). Control, but not MCI or AD, CSF T-tau levels were significantly positively correlated with the *TOMM40* promoter cg22024783 (Fig. 4b). Control, but not MCI or AD, CSF P-tau levels were significantly positively correlated with the *TOMM40* promoter cg22024783 (Fig. 4c). Control, MCI and AD, CSF $A\beta_{42}$, levels were not significantly correlated with the *APOE* CpG island cg05501958 (Fig. 4d). AD, but not controls, CSF T-tau levels were significantly positively correlated with the *APOE* CpG islandcg05501958 (Fig. 4e). Control, MCI and AD, CSF T-tau levels were not significantly correlated with the *APOE* CpG island cg05501958 (Fig. 4f). These results indicate that methylation of *TOMM40* promoter cg22024783 is positively correlated with AD-related biomarkers in controls, but in MCI and AD this positive correlation was lost. In addition, the *APOE* CpG island cg05501958 was positively correlated in AD for two AD-related biomarkers; T-tau and P-tau, but not $A\beta_{42}$. Taken together, these results suggest that PB methylation at the extended *APOE* locus is changed in AD. Cerebellum (CB) was also analyzed to demonstrate differences in DNA methylation between CB and HP as well as PB and all results are summarized in Table 2 and the Supplementary Tables.

Discussion

By most accounts, apoE protein is higher in cognitively normal control brain and cerebrospinal fluid (CSF) compared to AD and lowest in CSF and plasma from

Table 2 Result summary

				Disease Comparison Post-mortem Brain		HP *TOMM40* mRNA			HP *APOE* mRNA			Disease Comparison Peripheral Blood				Tissue Comparison						CSF Aβ42			CSF T-tau			CSF P-tau	
Genomic Context	ID	CpG	Genomic (HG19) Location	AD HP vs Controls HP	AD CB vs Controls CB	All	Controls	AD	All	Controls	AD	Controls vs MCI	Controls vs AD	Controls vs MCI & AD	MCI vs AD	Control HP vs Control Blood	AD HP vs AD Blood	Control CB vs Control HP	AD CB vs AD HP	Control CB vs Control Blood	AD CB vs AD Blood	Controls	MCI	AD	Controls	MCI	AD	Controls	MCI
TOMM40 CpG Island and Promoter	1	cg08267701	45393621	+																									
TOMM40 CpG Island and Promoter	2	cg22024783	45393916						+			+		+										+	+			+	
TOMM40 CpG Island and Promoter	3	cg27534894	45393925																										
TOMM40 CpG Island and Promoter	4	cg21549639	45394156																										
TOMM40 CpG Island and Promoter	5	cg12271581	45394330												+														
TOMM40 CpG Island and Promoter	6	cg19375044	45394343																				+						
TOMM40 CpG Island and Promoter	7	cg12540815	45394385																										
TOMM40 CpG Island and Promoter	8	cg06632829	45394476		+			+							+														
TOMM40 CpG Island and Promoter	9	cg12266551	45394624				+																						
TOMM40 CpG Island and Promoter	10	cg00441754	45394894																										
TOMM40 Intron 2	11	cg02613937	45395297																					+					
TOMM40 Intron 6	12	cg13447416	45398091					+		+										+	+		+						
TOMM40 3'UTR	13	cg14130141	45406886														+				+								
Intergenic	14	cg14123992	45407868	+												+	+	+	+	+	+		+						
Intergenic	15	cg04406254	45407945													+	+	+		+	+						+		
Intergenic	16	cg01032398	45408121															+	+	+	+								
APOE Promoter	17	cg26190885	45409005								+			+				+										+	
APOE Promoter	18	cg12049787	45409080	+								+			+	+				+	+								
APOE Promoter	19	cg08955609	45409353							+																			
APOE Promoter	20	cg18768621	45409440															+								+		+	
APOE Promoter	21	cg19514613	45409713													+	+			+	+								
APOE Intron 2	22	cg06750524	45409955														+	+											
APOE CpG Island Exon 4 - 3'UTR	23	cg16471933	45411802						+	+						+	+	+	+	+	+								+
APOE CpG Island Exon 4 - 3'UTR	24	cg05501958	45411873									+		+		+	+	+	+	+							+		
APOE CpG Island Exon 4 - 3'UTR	25	cg18799241	45412599			+						+		+		+	+	+	+										
APOC1 Promoter	26	cg00397545	45417526													+		+	+		+			+					
APOC1 Promoter	27	cg23270113	45417587					+				+				+	+	+											
APOC1 Promoter	28	cg09379229	45417668			+										+	+	+	+			+			+				
APOC1 Promoter	29	cg07773593	45417793													+	+	+	+	+	+								
APOC1 Promoter	30	cg13880303	45417814					+				+				+	+			+	+								
APOC1 Promoter	31	cg05644480	45418020													+	+	+	+	+	+			+					
APOC1 3'UTR	32	cg24795932	45422541																										
Intergenic	33	cg11911305	45428924								+																		
Intergenic	34	cg23184690	45429771													+	+			+	+								
Intergenic	35	cg08121984	45429870													+	+			+	+		+						
Intergenic	36	cg00587465	45429910																		+								
APOC1P1 Promoter	37	cg24084606	45430113					+								+	+			+	+								
Intergenic	38	cg08656316	45444199	+								+					+	+		+	+								
Intergenic	39	cg04766076	45444811													+											+		
Intergenic	40	cg06736138	45444860													+	+		+	+	+								
Intergenic	41	cg04401876	45445449														+	+		+	+								
APOC4-APOC2 Promoter	42	cg04347059	45445486			+										+	+	+		+	+				+		+		
APOC4-APOC2 Promoter	43	cg02912790	45445491													+	+	+		+	+								
APOC4-APOC2 Promoter	44	cg27353824	45445521													+	+	+		+	+	+			+				
APOC4-APOC2 Intron 1	45	cg25017250	45445693		+															+	+								
APOC4-APOC2 Exon 3	46	cg14723423	45448413			+										+	+			+	+						+		
APOC4-APOC2 Exon 3	47	cg22164781	45448680														+				+								+
APOC4-APOC2 Exon 3	48	cg10169327	45448959													+				+	+						+		
APOC4-APOC2 Exon 3	49	cg01958934	45449099													+	+	+	+	+	+								
APOC4-APOC2 Exon 3	50	cg13119609	45449297					+										+	+	+	+								
APOC4-APOC2 Exon 3	51	cg09555818	45449301	+				+										+	+	+	+								
APOC4-APOC2 Intron 3	52	cg25746394	45450501													+	+			+	+								
APOC4-APOC2 Exon 5	53	cg20090143	45452003																							+			+
Intergenic	54	cg22379747	45455191		+		+									+		+	+	+	+								

Multivariate regression for 54 CpGs was compared between AD and controls in post-mortem brain hippocampus (HP) and cerebellum (CB). Linear regression for each CpG (1–54) was analyzed for an association with *TOMM40* and *APOE* mRNA levels. Multivariate regression for 54 CpGs was compared between controls and MCI or AD in peripheral blood. Linear regression for each CpG (1–54) were analyzed for an association with CSF AD-biomarker levels. *p*-Values <0.050 are denoted as a plus sign (+)

individuals that carry the *APOE* ε4 allele [4, 19–21, 57–63]. In light of AD clinical trials that hope to modulate apoE levels, it is imperative to understand the complex regulatory region surrounding *APOE* including the methylation status of regional regulatory elements [24]. Since DNA methylation influences gene regulation [28–34] and since DNA methylation of *APOE* [39–43] has been described, but less is known about the surrounding complex regulatory structure, the aim of this investigation was to explore the DNA methylation status of the larger region surrounding the *APOE* gene and the relationship with tissue type, disease status, gene expression, and AD-related biomarkers.

Methylation results from HP, CB, and PB revealed differences in methylation between tissues and genomic regions (Fig. 2). These results are supported by previous reports that identified differences in methylation between brain and blood in AD [64, 65]. Two hypomethylated regions exist at the *TOMM40* and *APOE* promoters (Fig. 2) in all three tissues. Others have described hypomethylation at the *APOE* promoter [39–43] but to our knowledge methylation status of the *TOMM40* promoter has not been previously described. Interestingly, methylation of the *TOMM40* promoter is the only regulatory region evaluated here that did not significantly differ between HP, CB, and PB (Fig. 2; Table 2) in either controls or AD. A lack of differences in *TOMM40* promoter methylation between brain and blood may reflect similar methylation-related gene regulatory mechanisms of *TOMM40* in these two tissues.

In the CB, three CpG were significantly different between AD and controls in the *TOMM40* promoter *APOC4-APOC2* promoter and in the Intergenic region downstream from *APOC4-APOC2* (Fig. 2). Only the *TOMM40* promoter region replicated a significant difference in methylation between AD and control CB. Even though, CB DNA methylation in our cohort and the replication cohort showed few regional similarities, it is important to note that DNA methylation can vary by a multitude of factors, such as age, which is different between these two cohorts [28–34]. Therefore, it is difficult to interpret why there is only an overlap between our cohort and the replication for the *TOMM40* promoter in the CB (Supplementary Fig. 1B, C; Supplementary Table 7). HP methylation within the *TOMM40* promoter, *TOMM40-APOE* intergenic region, *APOE* promoter, *APOC1P1-APOC4* intergenic region, and *APOC4-APOC2* exon 3 were significantly different between AD and normal controls (Fig. 2). The *APOE* promoter CpG results in the HP are consistent with previous reports that identified differences between AD and control methylation of *APOE* [40, 42, 43], but to our knowledge the methylation of the surrounding CpG, outside of the *APOE* gene, including in the *TOMM40* promoter have not been characterized previously in AD.

Since methylation can impact gene expression levels, and methylation of both the *TOMM40* promoter and the *APOE* promoter was found to be associated with AD (Supplementary Fig. 1), both *APOE* and *TOMM40* levels were analyzed for an association between HP expression and methylation (Supplementary Fig. 2A, B). Interestingly, methylation of the *TOMM40* promoter was associated with both *APOE* and *TOMM40* levels. In contrast, only methylation of the *TOMM40* promoter, not methylation of the *APOE* promoter, was associated with *TOMM40* levels. Furthermore, *TOMM40* promoter methyation was associated with AD as well as HP *APOE* and *TOMM40* expression (Supplementary Figs. 1 and 2). In addition, a positive correlation between *APOE* transcript levels and *TOMM40* promoter methylation (cg22024783) as well as a negative correlation with *TOMM40* expression levels was observed (Fig. 3). Taken together, these results suggest that increasing methylation of the *TOMM40* promoter is associated with increasing *APOE* expression, but decreasing *TOMM40* expression. These results implicate methylation as a contributor to opposing *APOE* and *TOMM40* gene expression patterns. Others have described this phenomenom for other genes [66, 67], but to our knowledge this is novel information for the *APOE* and *TOMM40* genes. Interestingly, the *TOMM40* promoter cg12266551 was negatively correlated with *TOMM40* levels in AD, and positively correlated in controls, although non-significantly (Fig. 3), further suggesting that methylation may influence expression in AD.

Notably, tissue comparison analyses suggest no difference between tissues within the *TOMM40* gene implicating constitutive methylation of *TOMM40* across these tissues while other regional promoters showed a difference in methylation between tissues (Table 2). However, these results should be approached with caution as the sample size of the brain cohort was especially small and is therefore susceptible to false negatives. Other CpG, downstream of *APOE*, for example, within the *APOC1* and *APOC4-APOC2* promoters, were also correlated with *TOMM40* levels. Interestingly, methylation of the *APOE* CpG island was associated with *TOMM40* levels (Supplementary Fig. 2B). Consistent with this finding, we have previously observed that this genomic region within the *APOE* CpG island may function as a regulatory element that influences gene expression, including expression of *TOMM40* [41]. Taken together, these results suggest that further study is needed to understand the complex role of methylation on transcript levels in the brain at this locus.

Evaluation of peripheral blood (PB) DNA methylation changes between disease groups (e.g., MCI and AD compared to cognitively normal controls) revealed methylation

changes for CpG within the *TOMM40* promoter (CpG Island), the *APOE* promoter, the *APOE* exon 4 and 3′ UTR region (CpG island) and the *APOC1* promoter or the *APOC1P1-APOC4-APOC2* intergenic region (Supplementary Fig. 3A). In contrast, there was no association between methylation and AD compared to cognitively normal controls. DNA methylation in PB was significantly different between disease groups in our cohort and in the replication cohort (Supplementary Fig. 3A, B; Supplementary Table 7), suggesting that methylation changes in the blood are associated with AD pathogenesis. Since underlying AD pathology is reflected in CSF AD-related biomarkers in cognitively normal controls, MCI and AD, AD-related biomarkers were also evaluated to validate the association between *APOE* locus methylation and AD pathology. Interestingly, CSF $A\beta_{42}$ levels significantly correlated with: *TOMM40* promoter CpG methylation in AD and MCI, *TOMM40* intron 2 in AD, *TOMM40* intron 6 in MCI, *TOMM40-APOE* intergenic in MCI, *APOC1* promoter in AD and controls, *APOC1-APOC1P1* intergenic in MCI, *APOC4-APOC2* promoter in controls (Supplementary Fig. 4A), suggesting that CSF $A\beta_{42}$ levels may be related to methylation status upstream and downstream from *APOE*, but not within the *APOE* gene. These results are further supported by a negative correlation in AD, but opposite positive correlation in controls, between CSF $A\beta_{42}$ levels and methylation of *TOMM40* promoter cg22024783 (Fig. 4a). Furthermore, CSF T-tau and P-tau levels are positively correlated with percent methylation in controls, but not in AD or MCI, within the *TOMM40* promoter CpG (Fig. 4b, c). In support of these results, *TOMM40* expression in human PB has been reported as lower in AD compared to controls suggesting that *TOMM40* is down-regulated in AD blood and implicates disrupted *TOMM40* gene regulation in cells in the PB in AD patients [68–70]. Taken together, these results implicate disruption of methylation associated regulation of the *TOMM40* promoter in MCI and AD. In contrast, CSF T-tau (Supplementary Fig. 4B) and P-tau (Supplementary Fig. 4C) were associated with methylation all across this extended *TOMM40-APOE-APOC2* locus, including *APOE*, suggesting that CSF T-tau and P-tau levels may be related to methylation status across this locus, including the *APOE* promoter and the *APOE* CpG island. Interestingly, correlation analyses for a *APOE* CpG island CpG (cg05501958) does not show the strong opposing positive and negative correlations for CSF T-tau in MCI or AD, compared to controls, as seen in for the *TOMM40* promoter (Fig. 4e). In support of an *APOE* CpG island relationship with AD, a previous report describes significantly lower average methylation in AD across the *APOE* CpG island shores (outer regions) [43]. However, it is important to note that in present study only three *APOE* CpG island CpG sites were evaluated. Therefore, these results do not entirely reflect the levels of methylation across the entire CpG island as in this previous study [43].

A limitation of this exploratory study was small sample size. There were only twelve individuals analyzed in the brain cohort with the main goal to explore DNA methylation status of AD, compared to cognitively normal controls, at the *TOMM40-APOE-APOC2* locus. This small sample size may have contributed to false negatives and therefore missed important methylation differences between AD and cognitively normal controls in this post-mortem brain cohort. In addition, the DNA methylation analysis was limited by the specific CpG available on the array. Consequently, some important DNA methylation changes related to AD may have been missed. Furthermore, both the brain and blood consist of multiple cell types and from this analysis of whole tissues it is unclear which cells drive the methylation changes observed.

In conclusion, genomic regions that show methylation changes by tissue or disease are often located in regulatory regions, such as promoters or enhancers, and are associated with gene expression [26, 28, 30, 32, 71–74]. In this exploratory study, methylation changes by tissue or disease were identified within the *TOMM40-APOE-APOC2* region. Notably, regions outside of the *APOE* gene are differentially methylated according to disease state suggesting that in addition to *APOE*, methylation of other sites within the larger *TOMM40-APOE-APOC2* region, are changed in AD.

In summary, these results suggest that there is a relationship between *TOMM40-APOE-APOC2* regulatory region methylation status and gene expression in the brain as well as AD-related biomarkers in the blood. This suggests that DNA methylation may play a role in *APOE*-related pathogenesis in AD and implicates DNA methylation as a potential therapeutic target for modulating *APOE* gene expression in AD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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