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FEATURED ARTICLE

Genome-wide transcriptome analysis identifies novel dysregulated genes implicated in Alzheimer's pathology

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Abstract

Introduction: Abnormal gene expression patterns may contribute to the onset and progression of late-onset Alzheimer's disease (LOAD).

Methods: We performed transcriptome-wide meta-analysis (N = 1440) of blood-based microarray gene expression profiles as well as neuroimaging and cerebrospinal fluid (CSF) endophenotype analysis.

Results: We identified and replicated five genes (*CREB5*, *CD46*, *TMBIM6*, *IRAK3*, and *RPAIN*) as significantly dysregulated in LOAD. The most significantly altered gene, *CREB5*, was also associated with brain atrophy and increased amyloid beta (Aβ) accumulation, especially in the entorhinal cortex region. *cis*-expression quantitative trait loci mapping analysis of *CREB5* detected five significant associations ($P < 5 \times 10^{-8}$), where

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*Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in the analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

rs56388170 (most significant) was also significantly associated with global cortical Aβ deposition measured by [^{18}F]Florbetapir positron emission tomography and CSF Aβ_{1-42}. **Discussion:** RNA from peripheral blood indicated a differential gene expression pattern in LOAD. Genes identified have been implicated in biological processes relevant to Alzheimer's disease. *CREB*, in particular, plays a key role in nervous system development, cell survival, plasticity, and learning and memory.

KEYWORDS
Alzheimer's disease, amyloid β, ADNI, CREB5, imaging genetics, microarray gene expression

1 | INTRODUCTION

Late-onset Alzheimer's disease (LOAD) is a progressive neurodegenerative condition characterized by brain aggregation of amyloid beta (Aβ) into extracellular plaques, and hyperphosphorylated tau into neurofibrillary tangles.[1,2] Although these two pathological markers in addition to impaired cognitive function are used to definitively diagnose Alzheimer's disease (AD) post-mortem, neither marker is sufficient to cause AD.[3-5] Aβ positron emission tomography (PET) imaging has revealed that some individuals with normal cognitive performance display similar amyloid deposition to other individuals with mild cognitive impairment (MCI), suggesting that the amyloid hypothesis may not be sufficient to explain AD risk or progression.[5-7] Because early intervention is a goal for AD trials, there has been extensive research aimed at identifying markers of biological processes associated with AD risk or progression, especially efforts to develop more accurate ante-mortem measures of Aβ and tau.[8-11]

Although there are studies linking AD neuropathology and cognitive performance to dysregulated gene expression in brain tissue,[12-14] there are few data regarding AD-related gene expression in peripheral blood and none based on large-scale cohorts with neuroimaging and cerebrospinal fluid (CSF) biomarkers. Given that systemic factors such as inflammation, oxidative stress, and immune function are posited to play important roles in AD risk or progression, AD diagnosis and biomarkers for AD such as brain Aβ load or structural atrophy may be associated with differential expression of some genes in peripheral blood.

Neuroimaging genetics has been applied successfully in the past to obtain information about which genes are involved with specific AD-related pathological processes.[15-20] Expression of genes in peripheral blood can be associated not just with AD diagnosis, but with AD-related neuroimaging biomarkers such as atrophy or Aβ deposition in brain. We hypothesize that a genome-wide transcriptome meta-analysis will identify dysregulated genes in AD and that expression levels of the dysregulated genes will be also associated with AD-related neuroimaging biomarkers such as Aβ deposition and structural atrophy. Furthermore, we hypothesize that *cis*-expression quantitative trait loci (*cis*-eQTL) analysis of the differentially expressed genes will identify associated single nucleotide polymorphisms (SNPs) and that these SNPs will also be associated with AD-related endophenotypes including neuroimaging and CSF biomarkers.

2 | METHODS

2.1 | Study participants

All individuals used in the analysis were participants of the Alzheimer's Disease Neuroimaging Initiative (ADNI), AddNeuroMed, or Mayo Clinic Study of Aging (MCSA) cohorts. Informed consent was obtained for all subjects, and the study was approved by the relevant institutional review board at each data acquisition site.

2.1.1 | Alzheimer's Disease Neuroimaging Initiative (ADNI)

The ADNI initial phase (ADNI-1) was launched in 2003 to test whether serial magnetic resonance imaging (MRI), PET, other biological markers, and clinical and neuropsychological assessment could be combined to measure the progression of MCI and early AD. ADNI-1 has been extended in subsequent phases (ADNI-GO, ADNI-2, and ADNI-3) for follow-up of existing participants and additional new enrollments. Demographic information, raw scan data, apolipoprotein E (*APOE*) and whole-genome genotyping data, microarray gene expression data, neuropsychological test scores, and clinical information are publicly available from the ADNI data repository (http://www.loni.usc.edu/ADNI/).[21] A total of 661 ADNI participants (213 cognitively normal older adults (CN), 200 early MCI (EMCI), 145 late MCI (LMCI), and 103 AD) were available for analysis.

2.1.2 | AddNeuroMed

The AddNeuroMed study is a prospective and longitudinal multicenter collaboration for the discovery of novel biomarkers for AD. Data were collected from six medical centers across Europe. A

complete description of participant recruitment, selection criteria, and characterization is available in detail elsewhere.[22-24] A total of 674 AddNeuroMed participants included 208 MCI, 223 AD, and 243 CN.

2.1.3 | Mayo Clinic Study of Aging (MCSA)

The MCSA study was launched in 2004 to investigate the prevalence, incidence, and risk factors for MCI and dementia.[25] Study participants from this prospective population-based cohort are enrolled from the community of Olmsted County, MN, and followed longitudinally. Initially participants older than 70 years of age were included in the study; more recently participants older than 50 (since 2012) and older than 30 (since 2014) are being enrolled. Participants are evaluated and undergo neuropsychological assessment to determine a diagnosis of clinically normal, MCI, or dementia; a subset of the participants also undergo neuroimaging studies including MRI and PET. Biological samples: PAXgene blood, plasma, and/or CSF, were collected for consenting participants. A total of 105 MCSA samples were selected for this study, which includes 44 participants with a clinical diagnosis of AD and 61 clinically normal controls with PiB-PET Pittsburgh Compound-B [PiB] amyloid imaging that scored below the threshold for PiB-PET positivity (CN-PiB-negative).

2.2 | Gene expression profiling analysis

Gene expression profiling from peripheral blood samples collected using PAXgene tubes for RNA analysis was performed on the Affymetrix Human Genome U219 Array (www.affymetrix.com, Santa Clara, CA) for ADNI and on the Illumina Whole-Genome DASL assay (www.illumina.com, San Diego, CA) for AddNeuroMed and MCSA. All probe sets were mapped and annotated with reference to the human genome (hg19). Raw microarray expression values were pre-processed followed by standard quality control (QC) procedures on samples and probe sets.[26] Briefly, raw expression values were pre-processed using the robust multi-chip average normalization method.[27] We checked discrepancies between the reported sex and the sex determined from sex-specific gene expression data including *XIST* and *USP9Y*.[28] We also evaluated whether SNP genotypes were matched with genotypes predicted from gene expression data.[29] After QC, the RNA expression profiles contained 21,150 probes in ADNI.

2.3 | Imaging processing

As detailed in previous studies,[30-33] a widely employed automated MRI analysis technique (FreeSurfer V5.1) was used to process T_1-weighted structural MRI scans.[30,31] [^{18}F]Florbetapir PET scans were pre-processed as described previously and were intensity normalized by the whole cerebellum.[33] The normalization yielded standardized uptake value ratio images.

RESEARCH IN CONTEXT

1. Systematic review: The authors reviewed the literature using PubMed and meeting abstracts and presentations. We have cited several studies regarding Alzheimer's disease (AD)–related gene expression analysis in peripheral blood. However, none has used independent large-scale cohorts with blood-based gene expression profiles for replication and neuroimaging and cerebrospinal fluid (CSF) biomarkers for further analysis.
2. Interpretation: Our findings demonstrate that RNA from peripheral blood indicated a differential gene expression pattern in AD. Expression levels of the replicated dysregulated genes in AD were associated with AD-related neuroimaging and CSF biomarkers such as amyloid beta (Aβ) deposition and structural atrophy, and the dysregulated genes have been implicated in biological processes relevant to AD.
3. Future directions: Further research is needed to determine the specific roles of the replicated dysregulated genes in AD-related processes. Expression of these genes in peripheral blood should be investigated for the potential to enhance current biomarker measures of AD risk/progression.

2.4 | Genotyping and imputation

Participants were genotyped using several Illumina genotyping platforms. *APOE* genotyping was separately conducted using standard methods as described previously to yield the *APOE* ε4 allele defining SNPs (rs429358, rs7412).[21] As the cohorts (ADNI and AddNeuroMed) used different genotyping platforms, we imputed un-genotyped SNPs separately in each cohort using MACH and the 1000 Genomes Project data as a reference panel by following the Enhancing NeuroImaging Genetics through Meta-Analysis (ENIGMA) imputation protocol (http://enigma.usc.edu/wp-content/uploads/2012/07/ENIGMA2_1KGP_cookbook_v3.pdf).[34] Before the imputation, we performed standard sample and SNP QC procedures as described previously: (1) for SNP, SNP call rate < 95%, Hardy-Weinberg *P*-value $< 1 \times 10^{-6}$, and minor allele frequency < 1%; (2) for sample, sex inconsistencies, and sample call rate < 95%.[15-17,35] Furthermore, to prevent spurious association due to population stratification, we selected only non-Hispanic participants of European ancestry who clustered with HapMap CEU (Utah residents with Northern and Western European ancestry from the CEPH collection) or TSI (Toscani in Italia) populations using multidimensional scaling analysis (www.hapmap.org) in PLINK.[35,36] Imputation and QC procedures were performed as described previously.[15,16] After the imputation, we imposed an r^2 value equal to 0.30 as the threshold to accept the imputed genotypes.

2.5 | Statistical analysis

Statistical analysis of microarray data was performed using a linear regression model to evaluate differences in gene expression between AD and CN with age, sex, batch effects, and RNA integrity number (RIN) values as covariates. From the LIMMA software, we used the function lmFit to generate the fit, and the function eBayes to generate statistical significance values (https://doi.org/10.1007/0-387-29362-0_23).[37] Meta-analysis was performed using a fixed-effect, inverse-variance-weighted model in the METAL software (https://genome.sph.umich.edu/wiki/METAL_Documentation).[38] Significant associations were determined using false discovery rate (FDR) adjustment for multiple testing.

2.6 | Imaging genetics analysis

We further investigated the association of candidate gene expression levels identified from expression profiling analysis with structural and functional neuroimaging phenotypes by performing whole brain imaging genetics analyses. Multivariable analysis of cortical thickness and Aβ accumulation was performed to examine effects of gene expression levels on vertex-by-vertex and voxel-by-voxel bases, respectively. In MRI scans, the cortical thickness was calculated by taking the Euclidean distance between the gray and white boundary and the gray and CSF boundary at each vertex on the surface.[39] The SurfStat software package (www.math.mcgill.ca/keith/surfstat/) was used to perform a multivariable analysis of cortical thickness on a vertex-by-vertex basis using a general linear model (GLM) approach. GLMs were developed using age, sex, years of education, MRI field strength, and total intracranial volume as covariates. The processed [^{18}F]Florbetapir PET images were used to perform a voxel-wise statistical analysis across the whole brain using SPM8 (www.fil.ion.ucl.ac.uk/spm/). We performed a multivariable regression analysis using age and sex as covariates. Adjustment for multiple comparisons was performed using the random field theory (RFT) correction for whole brain surface-based analysis and FDR correction methods for whole brain voxel-based analysis.[40]

3 | RESULTS

Sample characteristics in each of the three data sets used in the discovery and replication analyses are presented in Table S1. This included 1440 non-Hispanic older adult participants of European ancestry (517 cognitively normal controls (CN), 553 individuals with MCI, and 370 with AD). The MCSA sample was older and had higher mean RIN values. The ADNI and AddNeuroMed cohorts consisted of CN and AD as well as MCI, although the MCSA cohort included only CN and AD. In addition, the ADNI and AddNeuroMed samples also had structural MRI scans.

3.1 | Genome-wide transcriptome analysis

In the discovery analysis using the ADNI cohort (N = 661), genome-wide comparison of AD with CN using 21,150 probes represented on the array after standard QCs was assessed and led to the identification of 26 significantly differentially expressed probes after controlling for multiple testing using FDR. Probes demonstrating significantly altered expression levels were shown in Supplementary Figure SF1 (a). In the Volcano plot, red open circles represented significantly differentially expressed probes in AD compared to CN. Eighteen probes were significantly upregulated and eight probes were significantly downregulated in AD. The 26 probes mapped to 23 corresponding genes. The top-hit upregulated gene was mitogen-activated protein kinase 14 *(MAPK14)*, followed by cAMP responsive element binding protein 5 (*CREB5*) and CD63 molecule (*CD63*). The leading downregulated gene was transmembrane protein 41A *(TMEM41A)*.

3.2 | Replication analysis

In total, 23 genes in the discovery were significantly differentially expressed in AD. However, of the 23 genes, 11 were observed in the replication data sets and were followed-up for replication and additional meta-analysis in the independent two cohorts (AddNeuroMed and MCSA; N = 571; 304 CN, 267 AD). Of 11 genes tested in the replication samples, five genes (*CREB5* [*P*-value = 1.29×10^{-6}], CD46 molecule [*CD46*; *P*-value = 8.19×10^{-6}], transmembrane BAX inhibitor motif containing 6 [TMBIM6; [*P*-value = 4.00×10^{-3}], interleukin 1 receptor associated kinase 3 [*IRAK3*; *P*-value = 1.81×10^{-4}], and RPA interacting protein [*RPAIN*; *P*-value = 2.37×10^{-3}]) were replicated and significantly differentially expressed in AD after Bonferroni correction for multiple comparisons (Table 1). Of these significant genes, the most significantly altered gene was *CREB5* (upregulated), followed by *CD46* and *IRAK3*. Supplementary Figure SF1 (b) displays the gene expression levels of *CREB5* across the continuum of AD. There is a significant increase in *CREB5* levels as the severity of AD increases and the expression of *CREB5* was altered from the early stages of disease. Of significantly downregulated genes, only one gene (*RPAIN*) was significantly replicated in the replication sample. In addition, in the combined discovery and replication sample, another gene (flotillin 1 [*FLOT1*]) showed evidence for association at genome-wide significance.

3.3 | Follow-up for association of *CREB5* gene expression levels with AD-related biomarkers

In order to investigate the effect of *APOE* ε4 status on levels of *CREB5*, the most significantly altered blood-based gene, we performed gene expression analysis after stratifying on *APOE* ε4 carrier status. *CREB5* was significantly upregulated in LOAD only in the *APOE* ε4 carrier group (β (standard error [SE]) = 0.25 [0.05], *P*-value = 1.37×10^{-5}).

TABLE 1 Meta-analysis of dysregulated genes

Gene	Discovery		Replication							
	ADNI		AddNeuroMed		MCSA		AddNeuroMed+MCSA		Meta-Analysis (ALL)	
	β (SE)	*P*-value	β (SE)	*P*-value	β (SE)	*P*-value	z-score	*P*-value	z-score	*P*-value
CREB5	0.18 (0.04)	5.03×10^{-6}	0.23 (0.05)	1.96×10^{-5}	0.12 (0.05)	2.18×10^{-2}	4.84	1.29×10^{-6}	6.61	3.90×10^{-11}
FLOT1	0.17 (0.04)	2.50×10^{-5}	0.07 (0.04)	5.56×10^{-2}	0.07 (0.06)	1.98×10^{-1}	2.28	2.26×10^{-2}	4.35	1.39×10^{-5}
CD46	0.18 (0.04)	2.93×10^{-5}	0.20 (0.04)	7.62×10^{-6}	0.06 (0.06)	3.31×10^{-1}	4.46	8.19×10^{-6}	6.07	1.26×10^{-9}
DUSP5	−0.20 (0.05)	3.20×10^{-5}	0.03 (0.04)	3.98×10^{-1}	−0.03 (0.04)	5.07×10^{-1}	0.48	6.32×10^{-1}	−2.10	3.59×10^{-2}
CD63	0.08 (0.02)	3.68×10^{-5}	0.02 (0.03)	5.68×10^{-1}	0.003 (0.061)	9.59×10^{-1}	0.54	5.91×10^{-1}	2.89	3.80×10^{-3}
PELO	0.15 (0.04)	7.49×10^{-5}	0.05 (0.03)	7.57×10^{-2}	0.04 (0.04)	3.97×10^{-1}	1.97	4.91×10^{-2}	3.94	8.07×10^{-5}
TMBIM6	0.06 (0.02)	9.80×10^{-5}	0.07 (0.02)	1.06×10^{-3}	−0.004 (0.022)	8.53×10^{-1}	2.88	4.00×10^{-3}	4.63	3.58×10^{-6}
IRAK3	0.23 (0.06)	9.96×10^{-5}	0.17 (0.05)	1.84×10^{-4}	0.03 (0.04)	3.93×10^{-1}	3.75	1.81×10^{-4}	5.33	9.96×10^{-8}
FKBP5	0.29 (0.07)	1.16×10^{-4}	0.13 (0.05)	3.71×10^{-3}	−0.15 (0.06)	1.17×10^{-2}	1.54	1.24×10^{-1}	3.54	4.06×10^{-4}
IL2R2	0.23 (0.06)	1.46×10^{-4}	0.08 (0.05)	1.41×10^{-1}	−0.04 (0.07)	6.08×10^{-1}	1.11	2.68×10^{-1}	3.16	1.60×10^{-3}
RPAIN	−0.10 (0.03)	3.28×10^{-4}	−0.10 (0.03)	1.55×10^{-3}	−0.01 (0.03)	6.74×10^{-1}	−3.04	2.37×10^{-3}	−4.58	4.58×10^{-6}

Five dysregulated genes were replicated in the replication sample.

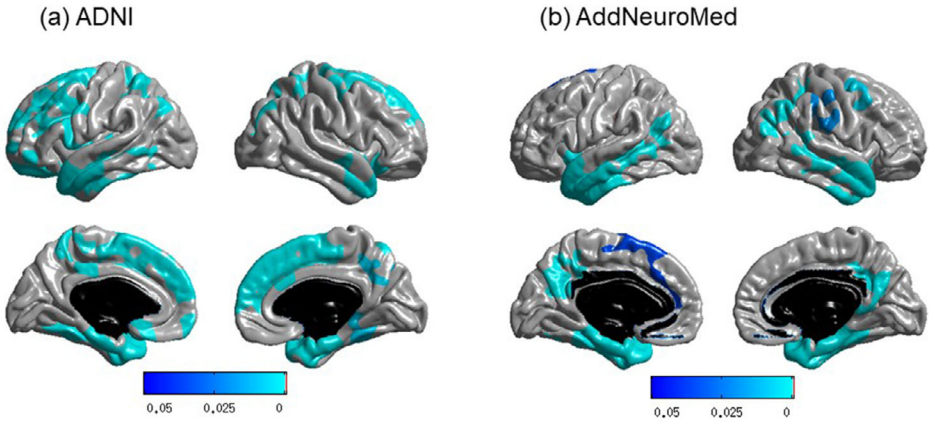

FIGURE 1 Association of *CREB5* gene expression levels with brain structure atrophy using whole brain surface-based analysis in two independent cohorts: (A) ADNI (discovery sample) and (B) AddNeuroMed (replication sample). Whole-brain cortical thickness analysis demonstrated the identification and replication of brain regions, especially entorhinal cortex, significantly associated with expression levels of *CREB5*. Statistical maps computed using SurfStat were thresholded using random field theory (RFT) as a multiple testing correction at *p*-corrected <0.05. The *P*-value indicates significant corrected *P*-values with the lightest blue color

We further investigated if levels of *CREB5* are also associated with AD-related biomarkers. Because *CREB* is a key component of learning and memory, we first performed an association of *CREB5* gene levels with cognitive performance using composite scores for memory and executive functioning and identified significant associations (β [SE] = −0.05 [0.01], *P*-value = 6.73×10^{-5} and β [SE] = −0.05 [0.01], *P*-value = 7.74×10^{-5} for composite scores for memory and executive functioning, respectively).[41-43] Increased expression levels of *CREB5* were associated with poor memory and cognitive performance.[44,45] We then performed whole brain surface-based analysis using cortical thickness on the brain surface on vertex-by-vertex bases measured from structural MRI scans. We used the ADNI and AddNeuroMed samples as the discovery and replication samples, respectively. In the ADNI sample including MCI patients, detailed whole-brain analysis identified clusters in a widespread pattern as significantly associated with expression of *CREB5* after adjusting for multiple comparisons using RFT (Figure 1 (a)). Individuals with higher expression levels showed greater atrophy in the bilateral frontal, parietal, and temporal lobes, especially including the entorhinal cortex. In the replication sample (AddNeuroMed) including MCI patients, highly significant clusters associated with *CREB5* gene expression levels were found in bilateral temporal cortical regions including the entorhinal cortex, where mean cortical thickness decreased as expression levels increased, which showed consistent patterns in the same brain regions in the independent two cohorts (Figure 1 (b)).

Next, as the Aβ peptide, one of the two main pathological hallmarks of AD, mediates synapse loss through the CREB signaling pathway,[44,45] in the ADNI cohort, we performed whole brain analysis using Aβ accumulation measured from [^{18}F]Florbetapir PET scans for association of expression of *CREB5* with Aβ load. The voxel-wise association results

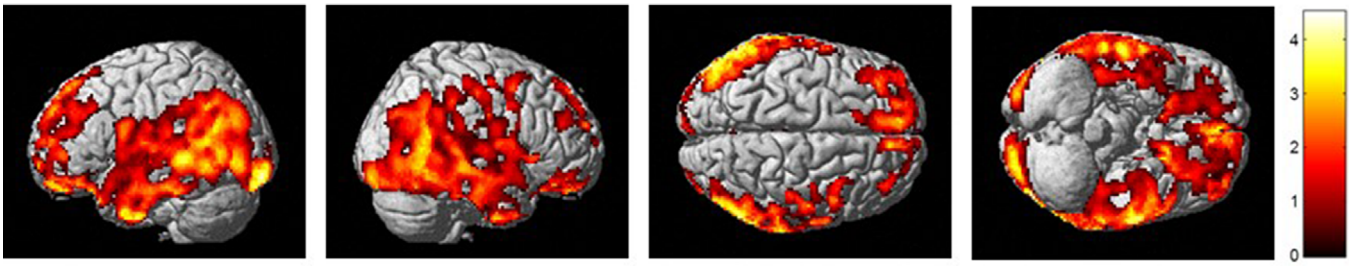

FIGURE 2 Association of *CREB5* gene expression levels with Aβ burden measured by [^{18}F]Florbetapir PET using whole brain analysis (FDR-corrected *P*-value <0.05)

were similar in association direction and regional distribution to those obtained from the cortical thickness analyses (Figure 2). Increased expression levels of *CREB5* were associated with increased Aβ accumulation in a widespread pattern, especially in the bilateral frontal, parietal, and temporal lobes after adjusting for multiple comparisons using FDR.

3.4 | Expression quantitative trait loci (eQTL) analysis of *CREB5*

We performed an eQTL analysis of the most significantly altered *CREB5* gene using SNPs imputed using the 1000 Genomes Project data as a reference panel in two independent cohorts (ADNI and AddNeuroMed). The analysis was limited to *cis*-eQTL, the SNPs of which were within 1 megabase (mb) distance from the transcription start or end sites of *CREB5*. In the ADNI sample, the eQTL mapping analysis of *CREB5* detected five significant associations with genome-wide significance at $P = 5 \times 10^{-8}$ (Figure 3 (a)).

Of these significant SNPs, rs56388170 within *CREB5* was most significantly associated with expression levels of *CREB5* (β [SE] = −0.15 [0.02], $P = 1.23 \times 10^{-16}$). Individuals carrying the minor allele of rs56388170 have lower expression levels of *CREB5*. In addition, the most significant SNP (rs56388170) was significantly replicated in the independent AddNeuroMed sample (β [SE] = −0.22 [0.05], $P = 3.90 \times 10^{-6}$; Figure 3 (b)). Because we showed that *CREB5* expression levels are associated with Aβ accumulation in the brain, we determined if rs56388170 is associated with Aβ load in AD. The most significant eQTL SNP (rs56388170) of *CREB5* was also significantly associated with global cortical Aβ load measured from [^{18}F] Florbetapir PET scans and CSF A$\beta_{1\text{-}42}$ with $P = .021$ (β [SE] = −0.008 [0.001]) and $P = .035$ (β [SE] = 0.013 [0.025]), respectively (Figure 4 (a) and (b)), where the minor allele of rs56388170 conferred decreases in cortical Aβ levels.

4 | DISCUSSION

This study identified and replicated five significantly differentially expressed genes as well as one marginally differentially expressed gene in AD in peripheral blood, five of which are upregulated (*CREB5*, *FLOT1*, *CD46*, *IRAK3*, and *TMBIM6*) and one of which is downregulated in AD (*RPAIN*). A literature search revealed no evidence of previous linkage of either *RPAIN* or *TMBIM6* with AD, suggesting that further exploration of the functions of these genes may yield novel information about AD processes. *RPAIN* is involved in transport of replication protein A (RPA), a eukaryotic single-stranded DNA binding protein with functions in DNA replication, repair, and recombination; *RPAIN* is an adaptor molecule that binds RPA and importin-β, and thus regulates RPA transport into the nucleus. With this function, *RPAIN*, via RPA, plays an important role in cell proliferation via cell cycle regulation.[46] *RPAIN* is ubiquitously expressed in numerous tissues including the brain.[47] Altered *RPAIN* expression could lead to reduced efficiency in DNA repair as well as cell cycle dysregulation, which has been observed previously in AD.[48] TMBIM6, also called Bax Inhibitor-1, is a transmembrane protein in the endoplasmic reticulum (ER). TMBIM6 is highly conserved and ubiquitously expressed in humans, and has been shown to play a role in numerous cellular pathways including ER stress, calcium imbalance, reactive oxygen species accumulation, and metabolic dysregulation. More specifically, this protein is involved in cellular calcium and pH homeostasis by mediating Ca(2+) efflux from the ER, and functions to protect the cell from ER stress-induced apoptotic cell death, although the exact molecular mechanism underlying this protein function is currently unclear.[49-51] Altered *TMBIM6* expression could dysregulate cellular response to stress, with interesting implications for AD susceptibility and progression. Upregulation of this protein, as observed in this study, may be a compensatory response to neuronal damage in AD. There is some evidence to suggest that TMBIM6 may play a protective role in the brain, both by protecting against apoptosis and potentially promoting neurogenesis during development. Of interest, TMBIM6 was reported to bind free presinilin 1; the resulting complex exhibited no proteolytic activity for Aβ, suggesting that this protein may play an important role in AD.[52] However, this work is based largely on animal and cellular models; more work remains to elucidate the functions of TMBIM6 in the human brain.

For three of the other genes significantly upregulated in this study (*CD46*, *IRAK3*, and *CREB5*), although there is evidence in the literature linking homologues or gene pathways to AD, there is a dearth of evidence directly linking *CD46*, *IRAK3*, and *CREB5* to AD. *CD46* has not been associated with AD; however, this ubiquitously expressed complement receptor protein has been shown to be necessary to the proper functioning of human cytotoxic CD8+ T cells in humans.[47,53] CD46 delivers co-stimulatory signals promoting cytotoxic CD8+ T cell

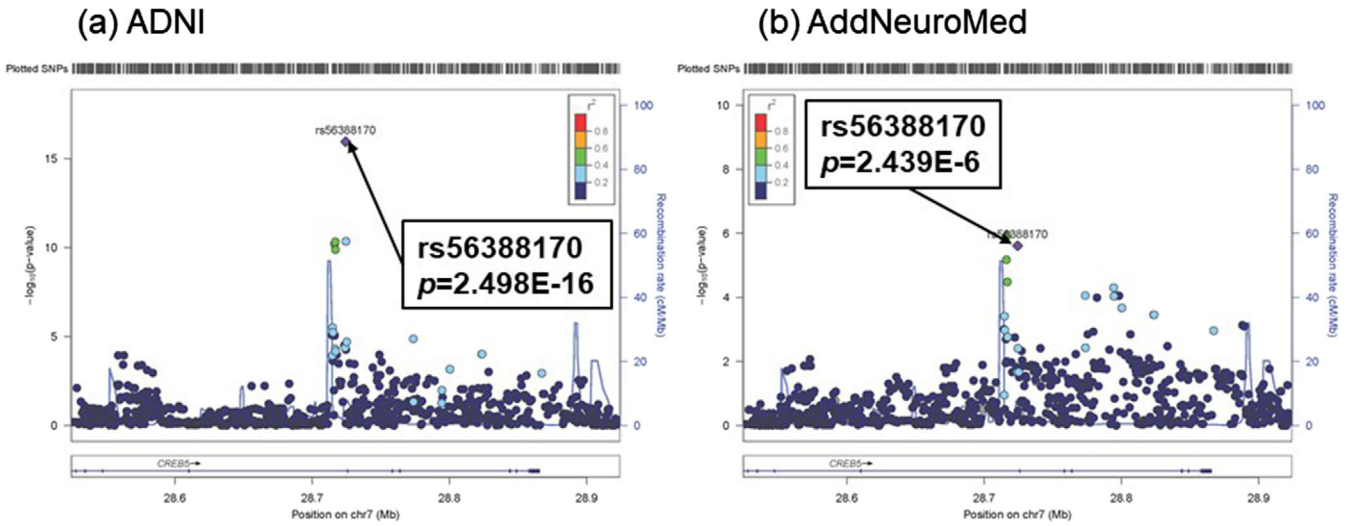

FIGURE 3 Results of *cis*-eQTL mapping analysis of *CREB5* using two independent cohorts: (A) ADNI (discovery sample) and (B) AddNeuroMed (replication sample). *cis*-eQTL mapping analyses of *CREB5* detected five significant associations with $P < 5 \times 10^{-8}$ in ADNI. The most significant *cis*-eQTL SNP (rs56388170) in ADNI was replicated in AddNeroMed. All SNPs are plotted based on their -$\log_{10}$*P*-values, NCBI build 37 genomic position, and recombination rates calculated from the 1000 Genomes Project reference data

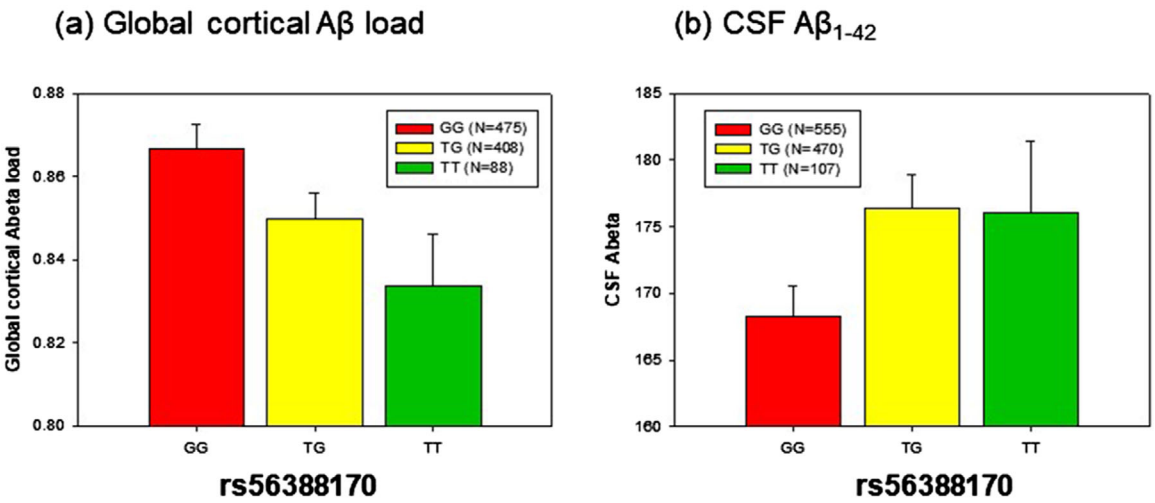

FIGURE 4 Association of the most significant *cis*-eQTL SNP (rs56388170) of *CREB5* gene expression levels with (A) global cortical Aβ levels measured by [^{18}F]Florbetapir PET and (B) CSF A$\beta_{1\text{-}42}$ in the ADNI. rs56388170 was significantly associated with global cortical Aβ load and CSF A$\beta_{1\text{-}42}$

activity; mutations, function, or regulation of this protein have been associated with viral infections as well as autoimmune diseases.[54-60] Although CD46 has not yet been associated with AD, the complement system has been significantly linked to AD. CD33 molecule *(CD33)*, another complement protein, is one of the top hits identified in the largest AD GWAS meta-analysis to date.[61] It has been postulated that the complement system works to increase inflammation, and that the AD-protective variant in *CD33* truncates the protein, resulting in reduced functions including cell signaling.[62] This suggests that lower expression of complement genes may have beneficial effects on the brain, aligning with our data showing lower *CD46* expression in controls compared to those with AD. *IRAK3*, another of the significantly upregulated genes, encodes an interleukin-1 receptor–associated kinase, which is ubiquitously expressed and functions in the Toll/IL-R immune signal transduction pathway.[47,63] Although there was no evidence from our literature search associating *IRAK3* with AD, other interleukin 1 receptor–associated kinases have been associated with pro-inflammatory processes in AD,[64,65] suggesting that these molecules play important roles in AD. Finally, CREB5 binds to cAMP response element (CRE) to function as a CRE-dependent transactivator. CREB proteins induce CRE-mediated gene transcription in response to cellular signaling.[66] cAMP signaling is involved in many cellular processes from cell growth and differentiation to gene transcription and protein expression.[67] Although *CREB5* has not been

linked specifically to AD, CREB family proteins are known to play important roles in synaptic strengthening and memory formation.[45] Increased CREB activity was observed in the peripheral blood of patients with mild AD or those with AD and depressive symptoms,[68] and increased CREB activity and dysregulation of CREB targets were observed in AD brain tissue compared to controls.[44] These previous results from the literature support the potential roles of *CD46*, *IRAK3*, and *CREB5* in AD.

In addition to these five genes, our meta-analysis of all three data sets identified *FLOT1* as significantly upregulated in AD. FLOT1 is a ubiquitously expressed protein that localizes to the caveolae, small domains on the inner cell membrane, and functions in vesicle trafficking and cell morphology pathways.[69-71] Flotillin proteins including FLOT1 have also been shown to play a role in endosomal sorting of beta-site amyloid precursor protein cleaving enzyme 1 (BACE-1), a protease involved in APP processing; depletion of flotilins results in stabilized BACE-1 and increased amyloidogenic processing of amyloid precursor protein.[72] Thus, it is possible that upregulation of flotilins may be a compensatory response by the cell to attempt to reduce amyloidogenic processing. Future analyses should investigate the expression of this gene in additional data sets for replication, as well as exploration of the impact of this protein on BACE-1 function in conjunction with AD risk and progression.

To extend interpretation of our findings and how the identified genes might impact the brain in LOAD, we also identified several larger AD brain tissue gene expression studies, and downloaded the lists of all differentially expressed genes. Although a comprehensive review of previous results was outside the scope of this study, we selected several of the larger, better-powered studies to discuss. We reviewed these lists of differentially expressed genes from these studies for presence of any of the five genes we identified as differentially expressed in AD in peripheral blood. The first study, of brain tissue collected from the current Mount Sinai Medical Center Brain Bank AD cohort, included expression data from 19 brain regions for 125 individuals across the AD spectrum, excluding any brain specimens that showed non-AD–related neuropathology. Gene expression was generated using the Human Genome (HG) Affymetrix U133A, U133B, or U133 Plus 2.0 microarrays. The researchers created high/normal/low groups based on AD traits: clinical dementia rating, Braak stage (tau pathology), Consortium to Establish a Registry for Alzheimer's Disease (CERAD; amyloid pathology), diagnostic certainty, amyloid plaque density mean, sum of neuritic plaque density estimates, and sum of neurofibrillary tangle density estimates. Differences between AD trait groups in at least one brain region were observed for *CREB5*, *TMBIM6*, and *RPAIN*.[13] The second study, which included brain tissue gene expression from 1647 specimens from LOAD and non-demented controls, investigated the association of gene networks with AD traits.[14] Study participants were recruited through the Harvard Brain Tissue Resource Center. Tissue specimens were from three brain regions: the dorsolateral prefrontal cortex, visual cortex, or cerebellum. Specifically, specimens were obtained from 549 brains, 376 of which were from LOAD patients, and 173 of which were from non-demented controls. In this data set, *CREB5* and *IRAK3* were included in networks of differentially expressed genes, but were not significantly associated with any of the AD traits analyzed. Another study, which included 87 individuals with AD and 74 controls, included lists of genes with differential expression associated with AD by brain regions.[12] The researchers analyzed differential expression in six brain regions: the entorhinal cortex, hippocampus, middle temporal gyrus, posterior cingulate cortex, superior frontal gyrus, and visual cortex; data were obtained from a study conducted by Liang et al. (2008) of gene expression from laser-capture microdissected non–tangle-bearing neurons in different brain regions.[73] The authors used a novel computational method to integrate gene expression information across brain regions, and identify a minimum common set of genes significantly associated with AD across brain regions. In this study, genes with differential expression associated with AD in at least one brain region included *CD46*, *TMBIM6*, *IRAK3*, *RPAIN*, and *FLOT1*. Finally, the last study by Ibanez et al. (2015) included two AD data sets: (1) hippocampal gene expression from nine controls and 15 individuals with AD,[74] and (2) brain tissue gene expression for varying numbers of individuals (all groups size range nine to 23) classified into each Braak stage (0-6) with 161 individuals total included.[75,76] This meta-analysis identified differential regulation associated with AD for *CREB5*, *IRAK3*, and *FLOT1*, although these comparisons did not survive multiple correction. To summarize, all of the six genes of interest from our study were identified in at least one of these prior studies of AD-related differential brain tissue gene expression. These findings support potential roles for brain function of the six genes of interest from our study of blood-based gene expression.

There are several plausible hypotheses regarding the mechanistic implications of the differential gene expression levels identified in blood. First, there may be genetic variants that drive expression levels of genes in both brain and blood. We have shown previously that many AD risk variants influence brain gene expression levels and are therefore likely to exert AD risk through their regulatory effects in the brain.[77-81] Some but not all eQTL have consistent effects across multiple tissue types.[82,83] It is possible that some or all of the peripheral differential expression detected in this study are a reflection of brain eQTL for those variants that have similar eQTL effects in blood. The associations of eQTL with *CREB5* levels, and the influence of the same eQTL with brain Aß levels, are consistent with this hypothesis.

The top genes identified in this study are implicated in pathways that are known to be important in AD pathophysiology such as synaptic processes (*CREB5*), endosomal sorting (*FLOT1*), immune system (*CD46*, *IRAK3*), and calcium homeostasis (*TMBIM6*). Hence, genetic variants that influence brain levels of these genes may also have an impact on these pathways and ultimately AD risk. Given the genetic heterogeneity of complex diseases, such as AD, eQTL, and differential gene expression, effects may be more readily detected than AD risk associations.[84] Therefore, expression profiling and eQTL studies provide a complementary approach to AD risk association in the identification of novel AD candidate genes and pathways.

Another plausible explanation of our results is that expression changes in blood may be reflective of cellular composition and other alterations in the brain that are downstream of disease pathology.

Although the majority of the expression levels captured by the blood measurements are likely driven by white blood cells, it is possible that these measurements also capture transcripts from other circulating cells, exosomal or cell free RNA. Single cell type, exosomal, and cell-free RNA-based studies are necessary to fully delineate the source of these peripheral expression levels. Given this possibility, another hypothesis is that peripheral gene expression levels are biomarkers of underlying disease pathology and/or its consequences, similar to blood Aß or cytokine measurements.[85,86]

These two hypotheses can be tested by various approaches, including multi-omics studies of large cohorts to discover eQTL, differential expression, and AD risk associations implicating the same gene; longitudinal blood expression studies in cohorts that also have concurrent brain pathology biomarkers, including neuroimaging and CSF; and finally in model systems where levels of these genes are perturbed and tested for AD-related outcomes.

This study has several limitations. First, this is an observational study, where we restricted our analysis to a non-Hispanic White population. Therefore, our findings may not be generalizable to other populations. It is important for future studies to investigate our findings using large community studies that include populations with greater diversity to determine if they translate to the broader population. Second, we performed gene expression analysis based on three independent studies using two different microarray platforms; despite this, our results were replicable results across these cohorts, which indicates that our findings are unlikely to be driven by technical artefacts. In addition, we performed a meta-analysis of three data sets instead of a mega-analysis. Third, the diagnosis was based largely on clinical criteria without neuropathology confirmation, which is a common limitation for ante-mortem studies. We addressed this by using AD-related endophenotypes measured by multimodal neuroimaging and CSF biomarkers. Furthermore, we explored the findings from brain tissue studies using neuropathologically diagnosed cases. Finally, our study is cross-sectional and it would be important in the future to use a larger prospective cohort study to determine the potential role of the identified genes.

Overall, six genes identified in this study may have the potential to provide further insight into the biological mechanisms underlying AD risk and progression. Further study is required to determine the specific roles of *CREB5*, *CD46*, *TMBIM6*, *IRAK3*, *RPAIN*, and *FLOT1* in AD-related processes. Expression of these genes in peripheral blood should also be investigated for potential to enhance current biomarker measures of AD risk/progression.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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