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RESEARCH ARTICLE

Identification of an *APOE* ε4–specific blood-based molecular pathway for Alzheimer's disease risk

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[♯]Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (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 analysis or writing of this report. A complete listing of ADNI

Abstract

INTRODUCTION: The precise apolipoprotein E (*APOE*) ε4-specific molecular pathway(s) for Alzheimer's disease (AD) risk are unclear.

METHODS: Plasma protein modules/cascades were analyzed using weighted gene co-expression network analysis (WGCNA) in the Alzheimer's Disease Neuroimaging Initiative study. Multivariable regression analyses were used to examine the associations among protein modules, AD diagnoses, cerebrospinal fluid (CSF) phosphorylated tau (p-tau), and brain glucose metabolism, stratified by *APOE* genotype.

RESULTS: The Green Module was associated with AD diagnosis in *APOE* ε4 homozygotes. Three proteins from this module, C-reactive protein (CRP), complement C3, and complement factor H (CFH), had dose-dependent associations with CSF p-tau and cognitive impairment only in *APOE* ε4 homozygotes. The link among these three proteins and glucose hypometabolism was observed in brain regions of the default mode

investigators can be found at: http://adni..usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf

network (DMN) in *APOE* ε4 homozygotes. A Framingham Heart Study validation study supported the findings for AD.

DISCUSSION: The study identifies the *APOE* ε4–specific CRP–C3–CFH inflammation pathway for AD, suggesting potential drug targets for the disease.

KEYWORDS

Alzheimer's disease, age-related macular degeneration, amyloid beta peptide, apolipoprotein E, cerebrospinal fluid phosphorylated tau, cognitive impairment, complement C3, complement factor H, C-reactive protein, hypometabolic convergence index, positron emission tomography

Highlights

- Identification of an APOE ε4 specific molecular pathway involving blood CRP, C3, and CFH for the risk of AD.
- CRP, C3, and CFH had dose-dependent associations with CSF p-Tau and brain glucose hypometabolism as well as with cognitive impairment only in APOE ε4 homozygotes.
- Targeting CRP, C3, and CFH may be protective and therapeutic for AD onset in APOE ε4 carriers.

1 | BACKGROUND

The apolipoprotein E (*APOE*) ε4 allele is a major genetic risk factor for Alzheimer's disease (AD), in a manner that heightens risk in a dose-dependent manner compared to persons with the common ε3/ε3 genotype.[1] Although *APOE* ε4 is related to aggregation or clearance of amyloid beta (Aβ), a component of AD pathology,[2] the precise molecular pathway by which *APOE* ε4 increases AD risk is not fully understood. Recent studies have suggested that blood C-reactive protein (CRP) could play a role as a mediating factor for AD risk among ε4 carriers.[3] CRP is a key protein involved in the cascade of events leading to peripheral infection and chronic low-grade inflammation.[4] It is unclear whether other blood cascade proteins co-expressed with CRP are also involved in the ε4-mediated pathological processes leading to AD. Identifying and characterizing proteins that interact with the *APOE* genotype could benefit drug development in line with a precision medicine approach for preventing and treating AD.

Based on our previous findings indicating that CRP is associated with AD risk in ε4 carriers in humans,[5,6] the goal of this study was to determine which other proteins in the biological pathway containing CRP would also modify the association of *APOE* genotype with AD-related traits including Aβ, phosphorylated tau (p-tau), and brain glucose hypometabolism, and cognitive performance. To approach this goal, we evaluated data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) study[7] to identify data-driven molecular modules (the clusters of plasma proteins) for AD risk in ε4 carriers, and validated the top-ranked findings in the Framingham Heart Study (FHS) cohort.

2 | METHODS

2.1 | Participants and study design

Data used in the preparation of this article were obtained from the ADNI database (adni.loni.usc.edu). The ADNI was launched in 2003 as a public–private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early AD. The study subjects included 566 ADNI-1 participants (baseline enrollment date: 2005 to 2007) who had clinical information, *APOE* genotype data, baseline plasma protein and cerebrospinal fluid (CSF) measurements, (18)F-fluorodeoxyglucose positron emission tomography (FDG-PET) data, and longitudinal measurements of cognitive function over 2 years. The study design is illustrated in Figure S1 in supporting information.

2.2 | Cognitive assessment and clinical diagnoses

Scores from the Mini-Mental State Examination (MMSE)[8] and Alzheimer's Disease Assessment Scale–Cognitive Subscale (ADAS including Delayed Word Recall and Number Cancellation) were measured at baseline, 6 to 12 (1 year), and 18 to 24 (2 years) months

after the plasma protein measurements. Diagnoses of cognitive normal (CN), MCI, and AD dementia were assigned using established criteria.[9]

2.3 | Plasma proteins

The protocol for quantifying plasma analytes in ADNI participants is described elsewhere.[10] Briefly, plasma aliquots (0.5 mL) under fasting condition were stored at −80°C. Plasma protein markers (190 analytes) were measured by Rules Based Medicine, Inc. (RBM) using the Human Discovery Multi-Analyte Profile (MAP) 1.0 panel and a Luminex 100 platform. After excluding 44 protein analytes with no values, data for 146 proteins were used in this study.

2.4 | Measurement of CSF biomarkers

Levels of Aβ_{42} and p-tau181 in CSF were measured using the multiplex xMAP Luminex platform and electrochemiluminescence immunoassays (lot# P09 for Aβ1-42 and P02 for p-tau181).[11] CSF Aβ1-42 in CSF and Phospho-Tau(181P) are widely used as biomarkers for AD diagnosis and research.[12]

2.5 | PET with (18)F-fluorodeoxyglucose

Brain glucose metabolism was examined using FDG-PET. Details of the PET image processing protocol and generation of the hypometabolic convergence index (HCI), a measure of the extent to which the pattern and magnitude of hypometabolism correspond to that observed in patients with a clinical diagnosis of AD, were previously published.[13] and are available online (https://adni.bitbucket.io/reference/baipetnmrc.html). We obtained the FDG scores at different brain regions from the ADNI dataset.

2.6 | Statistical analyses

Protein analytes were log-transformed to approximate a normal distribution. Protein modules were identified by gene co-expression network analysis using weighted gene co-expression network analysis (WGCNA) software.[14,15] Associations of protein modules with AD-related traits in the entire sample were evaluated using linear regression models including covariates for age, sex, and education (Figure S2C in supporting information). Four modules associated with AD diagnosis and one module containing *APOE* (Brown) were chosen for further analysis (Figure 1B). Demographics and WGCNA scores of the five modules were compared across *APOE* genotype groups defined by the number of ε4 alleles (0 = ε2/ε2, ε2/ε3, ε3/ε3; 1 = ε2/ε4, ε3/ε4; 2 = ε4/ε4) using one-way analysis of variance (ANOVA) for continuous variables and chi-square tests for categorical variables. The association of modules with AD-related traits within *APOE* genotype groups was evaluated using regression models defined above. Individual proteins within modules that had *APOE* genotype–dependent associations with AD-related traits were also tested for association with AD-related traits. Analyses were performed using R (The R Foundation for Statistical Computing v4.2.1; https://www.eea.europa.eu/data-and-maps/indicators/oxygen-consuming-substances-in-rivers/r-development-core-team-2006). A two-sided test with a significance threshold of $P < 0.05$ was used for all analyses. Bonferroni correction for multiple testing were applied to results from these analyses.

To understand the protein pathways for AD observable in blood, we selected all proteins from the five WGCNA modules described above (Figure 1B) for pathway enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery.[16] Due to the

RESEARCH IN CONTEXT

1. **Systematic review**: The authors reviewed the literature using PubMed sources. The apolipoprotein E (*APOE*) ε4 allele has been established as a significant genetic risk factor for Alzheimer's disease (AD), increasing the risk in a dose-dependent manner compared to individuals with the common ε3/ε3 genotype. However, the precise molecular pathway through which it increases AD risk is not fully understood. Recent studies have suggested that blood C-reactive protein (CRP) may play a role as a mediating factor for AD risk among *APOE* ε4 carriers. But it remains unclear whether other blood cascade proteins co-expressed with CRP are also involved in the pathological processes leading to AD in individuals with the *APOE* ε4 genotype. These relevant referenced are appropriately cited.
2. **Interpretation**: Our findings led to an integrated hypothesis regarding other proteins within the biological pathway containing CRP that may modify the association of AD-related traits in an *APOE* genotype dependent manner. We found that (1) the role of *APOE* ε4–specific molecular pathway involving blood CRP, complement C3, and complement factor H (CFH(for the risk of AD; (2) each of the proteins, CRP, C3, and CFH, demonstrated dose-dependent associations with cerebrospinal fluid phosphorylated tau, brain glucose hypometabolism, and cognitive impairment specifically in *APOE* ε4 homozygotes. The hypothesis and the results of this study are consistent with non-clinical and clinical findings currently in the public domain.
3. **Future directions**: The findings point to the future direction that targeting CRP, C3, and CFH may have protective and therapeutic implications for delaying the onset of AD in individuals with the *APOE* ε4 genotype.

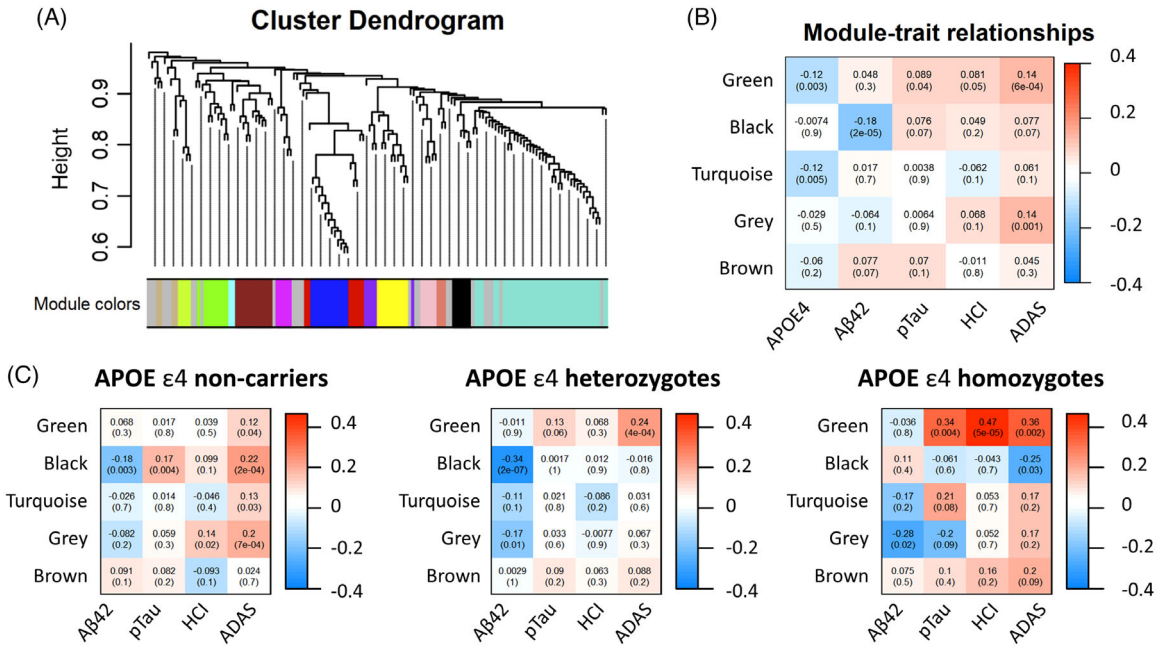


FIGURE 1 Characterization of plasma protein clusters and their associations with AD biomarkers in an *APOE* genotype dependent manner. (A) WGCNA was performed using 146 plasma proteins for protein clustering, and protein modules were detected from the dendrogram using a dynamic tree-cutting algorithm. A total of 15 modules were identified, and labeled by different colors. (B) Based on their associations with AD diagnosis (Figure S2C in supporting information) or having *APOE* protein, five modules were chosen for their relationships with the AD traits. Module eigengenes (MEs) and their correlations with AD biomarkers, including *APOE* ε4 genotype, CSF Aβ42, and p-tau, brain HCI and ADAS were determined by using Pearson correlation in the whole sample. The correlation coefficients (with a *P*-value in parentheses) are shown. Blocks were painted with different colors, representing the degrees of significance, that is, red, positive and blue, negative. (C) Stratification based on the number of *APOE* ε4 alleles, non-carriers (ε4 = 0), heterozygotes (ε4 = 1), and homozygotes (ε4 = 2), was performed and the same correlation analyses as in (B) were conducted in each *APOE* genotype group. The Green module's associations except CSF Aβ42 were found to be *APOE* ε4 homozygote genotype dependent with statistical significance; the Black module's associations were modest in the *APOE* ε4 non-carrier genotype. Aβ, amyloid beta; AD, Alzheimer's disease; ADAS, Alzheimer's Disease Assessment Scale-Cognitive Subscale; *APOE*, apolipoprotein E; CSF, cerebrospinal fluid; HCI, hypometabolic convergence index; p-tau, phosphorylated tau; WGCNA, weighted gene co-expression network analysis.

limited size of the modules, we were unable to evaluate pathway enrichment among individual modules. Overrepresented functions with significant enrichment ($P < 0.05$) were identified based on biological process Gene Ontology terms, Kyoto Encyclopedia of Genes and Genomes pathways, and Reactome pathway analyses. Proteins from the Green module were further evaluated using the STRING database (https://string-db.org/)[17] to explore other protein–protein interactions. The resulting protein–protein interaction network was visualized using Cytoscape (version 3.9.1),[18] and functional modules within the network were identified using the Mosaic app.[19]

3 | Replication and extension of top-ranked protein findings

To replicate and validate the pathway involved in CRP, complement C3 (C3), and complement factor H (CFH), we analyzed data for all proteins in the top-ranked module in the FHS Offspring cohort obtained from Exam 5 (1991 to 1995). Details about aptamer-based proteomic profiling in FHS were previously reported.[20,21] Due to the limited number of AD cases among these FHS participants, most of whom had not reached the age when disease symptoms typically occur (mean age ± standard deviation = 55 ± 10, range = 29 to 82 years), there was insufficient statistical power to conduct cross-sectional analyses. Therefore, we used a longitudinal design to investigate the association between baseline protein measures and AD incidence using proportional hazards analysis applied to the entire sample and separately to *APOE* ε4 non-carriers and carriers. Hazard ratios (HRs) and their 95% confidence intervals (CIs) were estimated in models including covariates for age, sex, and education. Models for the entire sample also included a term for the interaction of ε4 carrier status with protein level.

4 | RESULTS

4.1 | Characterization of plasma proteins clusters and their associations with AD traits

Complete *APOE* genotype data and baseline plasma protein measurements were available for 566 ADNI participants. These participants

TABLE 1 Demographic information, cognitive diagnoses, and the AD biomarkers stratified by *APOE* ε4 allele status in the ADNI study.

		APOE ε4 carrier status			
Characteristics mean ± SD or *n* (%)	Total *n* = 566	No ε4 allele (ε4 = 0) *n* = 274	One ε4 allele (ε4 = 1) *n* = 222	Two ε4 alleles (ε4 = 2) *n* = 70	*P* values
Age (years)	74.8 ± 7.4	75.7 ± 7.8	74.9 ± 6.7	70.9 ± 6.4	<0.001
Female (%)	215 (38.0)	105 (38.3)	81 (36.5)	29 (41.4)	0.75
Education (years)	15.5 ± 3.0	15.7 ± 3	15.4 ± 3.2	15.5 ± 2.6	0.39
MMSE scores	26.5 ± 2.4	27.0 ± 2.2	26.2 ± 2.4	25.7 ± 2.4	<0.001
ADAS scores	19.7 ± 8.2	17.2 ± 8.2	21.7 ± 7.7	22.8 ± 7.1	<0.001
Diagnosis					
CN	58 (10.2)	53 (19.3)	5 (2.3)	0 (0)	
MCI	396 (70.0)	185 (67.5)	164 (73.9)	47 (67.1)	<0.001
AD	112 (19.8)	36 (13.1)	53 (23.9)	23 (32.9)	
CSF markers	***n* = 340**	***n* = 168**	***n* = 129**	***n* = 43**	
Aβ42, pg/mL	878 ± 456	1120 ± 457	692 ± 325	483 ± 153	<0.001
t-tau, pg/mL	311 ± 137	278 ± 124	342 ± 150	346 ± 112	<0.001
p-tau, pg/mL	30.6 ± 15.4	26.3 ± 13.8	34.5 ± 16.4	35.6 ± 14.0	<0.001
Brain PET summaries	***n* = 290**	***n* = 135**	***n* = 116**	***n* = 39**	
HCI	15.1 ± 6.92	13.9 ± 7.41	15.9 ± 6.45	16.8 ± 5.85	0.02
WGCNA modules*					
Green	0 ± 1.0	0.14 ± 0.96	−0.13 ± 1.00	−0.13 ± 1.0	0.01
Black	0 ± 1.0	−0.02 ± 1.0	0.06 ± 0.98	−0.11 ± 1.0	0.63
Turquoise	0 ± 1.0	0.11 ± 1.1	−0.07 ± 0.90	−0.22 ± 0.87	0.05
Gray	0 ± 1.0	0.01 ± 1.0	0.03 ± 0.98	−0.13 ± 0.95	0.70
Brown	0 ± 1.0	0.07 ± 1.0	−0.06 ± 0.98	−0.07 ± 0.98	0.48

Note: ADNI participants were stratified into three groups based on the presence of no versus one versus two *APOE* ε4 alleles.
Abbreviations: AD, Alzheimer's disease; ADAS, Alzheimer's Disease Assessment Scale-Cognitive Subscale; ADNI, Alzheimer's Disease Neuroimaging Initiative; ANOVA, analysis of variance; *APOE*, apolipoprotein E; CN, cognitively normal; CSF, cerebrospinal fluid; HCI, hypometabolic convergence index; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; PET, positron emission tomography; p-tau, phosphorylated; SD, standard deviation; t-tau, total tau
*All WGCNA module scores were the standardized *z* scores. Mean ± SD was reported for continuous variables, while *n* (%) was used for binary variables. For the continuous variables, one-way ANOVA was used to determine whether there are any statistically significant differences between the means of three *APOE* ε4 groups, that is, non-carriers (ε4 = 0), heterozygotes (ε4 = 1), and homozygotes (ε4 = 2), while the chi-square test (χ^2) was used to examine the differences between categorical variables in the same population. *P* values for the comparisons of three *APOE* groups are shown.

had a mean age of 74.8 ± 7.4 years, and 215 of them (38%) were female (Figure S1). Diagnostically, 58 were CN (10.2%), 396 had MCI (70.0%), and 112 had AD (19.8%; Table 1). Further analysis showed that increases in the number of *APOE* ε4 alleles were associated with younger age, higher likelihood of having MCI or AD, lower levels of CSF Aβ42, higher levels of CSF total tau and p-tau, and higher HCI scores. There was no difference of body mass index across the *APOE* genotypes.

WGCNA analysis revealed 15 modules with distinct co-expressed protein networks (Figure 1A; Figure S2A). Four modules (color-labeled as Green, Black, Turquoise, Gray) were associated with AD diagnosis; however, after Bonferroni correction ($P < 0.003$), only the Green and Gray modules remined significant (Figure S2C, last column). Associations involving AD-related traits were evaluated in these four modules in addition to the Brown module, which contained *APOE* (Figure 1B). All modules except Brown were modestly associated with at least one AD-related trait in the total sample, most notably CSF Aβ42 with the Black module ($P = 0.00002$), and ADAS with the Green ($P = 0.0006$) and Gray ($P = 0.001$) modules. Interestingly, although the Brown module contained the apoE protein, *APOE* genotype was not associated with the Brown module, but rather with the Green and Turquoise modules.

Analysis of co-expressed gene networks within and across *APOE* genotype groups revealed that protein co-expression in the Green module in ε4 homozygotes and the Black module in the group lacking ε4 were associated with at least three of the four AD traits (Figure 1C and Figure S2C), whereas protein co-expression in the Turquoise, Gray, and Brown modules showed little evidence of association with the AD-related traits in any of the *APOE* genotype groups.

TABLE 2 The associations between individual proteins from the green module and AD-related traits.

			APOE ε4 noncarriers			*APOE ε4* heterozygotes			*APOE ε4* homozygotes		
X	Y	Time	*n*	Beta ± SE	P values	n	Beta ± SE	P values	*n*	Beta ± SE	*P* values
CRP	p-tau	Baseline	168	0.00 ± 0.03	0.06	129	0.01 ± 0.03	0.87	43	0.08 ± 0.04	**0.03**
		1 year	132	0.00 ± 0.03	**0.0005***	104	0.01 ± 0.03	0.76	34	0.13 ± 0.06	**0.04**
		2 years	47	0.00 ± 0.05	0.02	29	0.00 ± 0.07	0.79	8	0.19 ± 0.19	0.41
	HCI	Baseline	135	0.03 ± 0.03	0.40	116	0.00 ± 0.03	0.57	39	0.13 ± 0.04	**0.001***
		1 year	117	0.02 ± 0.04	0.58	96	0.00 ± 0.04	0.40	34	0.11 ± 0.06	0.10
		2 years	97	0.02 ± 0.04	0.59	81	0.00 ± 0.04	0.27	26	0.12 ± 0.08	0.15
	ADAS	Baseline	273	0.00 ± 0.03	0.85	218	0.01 ± 0.02	0.47	70	0.07 ± 0.03	**0.03**
		1 year	246	0.01 ± 0.04	0.85	195	0.01 ± 0.03	0.64	63	0.11 ± 0.05	**0.02**
		2 years	208	0.04 ± 0.04	0.34	170	0.00 ± 0.03	0.95	53	0.09 ± 0.05	0.08
C3	p-tau	Baseline	168	0.00 ± 0.18	0.86	129	0.12 ± 0.22	0.58	43	0.19 ± 0.32	0.56
		1 year	132	0.00 ± 0.21	0.13	104	0.21 ± 0.24	0.37	34	0.19 ± 0.44	0.67
		2 years	47	0.00 ± 0.35	0.99	29	0.31 ± 0.54	0.57	8	2.40 ± 0.38	**0.008**
	HCI	Baseline	135	0.00 ± 0.19	0.55	116	0.00 ± 0.20	0.49	39	0.64 ± 0.26	**0.02**
		1 year	117	0.00 ± 0.20	0.70	96	0.00 ± 0.25	0.55	34	0.72 ± 0.33	**0.04**
		2 years	97	0.00 ± 0.26	0.66	81	0.00 ± 0.32	0.40	26	0.91 ± 0.48	0.07
	ADAS	Baseline	273	0.16 ± 0.16	0.32	218	0.30 ± 0.13	**0.02**	70	0.69 ± 0.19	**0.0007***
		1 year	246	0.13 ± 0.21	0.55	195	0.32 ± 0.17	0.07	63	0.90 ± 0.28	**0.002***
		2 years	208	0.40 ± 0.26	0.13	170	0.48 ± 0.19	**0.02**	53	0.84 ± 0.32	**0.01**
CFH	p-tau	Baseline	168	0.00 ± 0.09	0.79	129	0.05 ± 0.11	0.66	43	0.38 ± 0.14	**0.01**
		1 year	132	0.00 ± 0.10	0.39	104	0.00 ± 0.11	0.99	34	0.52 ± 0.17	**0.005***
		2 years	47	0.00 ± 0.20	0.52	29	0.00 ± 0.22	0.75	8	0.73 ± 0.52	0.25
	HCI	Baseline	135	0.09 ± 0.10	0.35	116	0.00 ± 0.09	0.63	39	0.40 ± 0.12	**0.002***
		1 year	117	0.12 ± 0.11	0.28	96	0.00 ± 0.10	0.34	34	0.47 ± 0.14	**0.002***
		2 years	97	0.08 ± 0.13	0.54	81	0.00 ± 0.13	0.88	26	0.42 ± 0.19	**0.04**
	ADAS	Baseline	273	0.09 ± 0.08	0.25	218	0.02 ± 0.06	0.77	70	0.25 ± 0.11	**0.02**
		1 year	246	0.10 ± 0.10	0.33	195	0.00 ± 0.08	0.99	63	0.37 ± 0.14	**0.01**
		2 years	208	0.19 ± 0.12	0.13	170	0.03 ± 0.10	0.77	53	0.38 ± 0.14	**0.01**

Note: ADNI participants were divided into three groups based on the number of *APOE ε4* alleles: non-carriers (ε4 = 0), heterozygotes (ε4 = 1), and homozygotes (ε4 = 2). Linear regression models were used in each genotype group to examine the relationships among individual proteins from the green module and the AD traits after adjusting for sex, age, and education. The relationship among plasma CRP, C3, or CFH and the AD traits, for example, CSF p-tau, HCI, or ADAS, measured at baseline, follow-ups 1 year, and 2 years, were shown. To account for multiple comparisons, we treated the nine proteins from the Green module as independent tests, and applied the Bonferroni correction by adjusting the *P*-value threshold to 0.05/9 = 0.006.

*Red indicates *P* values significant after Bonferroni correction. Blue indicates $P < 0.05$.

Abbreviations: AD, Alzheimer's disease; ADAS, Alzheimer's Disease Assessment Scale-Cognitive Subscale; ADNI, Alzheimer's Disease Neuroimaging Initiative; *APOE*, apolipoprotein E; C3, complement C3; CFH, complement factor H; CRP, C-reactive protein; HCI, hypometabolic convergence index; p-tau, phosphorylated tau.

4.2 | Associations of proteins in the Green module with AD biomarkers and brain glucose metabolism are *APOE* genotype dependent

Of the nine proteins (CRP, CFH, C3, AAT, HP, P SAP, TBG, and vitamin K–dependent proteins [VKDPs]) in the Green module (Table S1 in supporting information; Figure S2A), CRP, C3, and CFH levels were generally nominally associated with CSF p-tau, HCI, and ADAS in ε4 homozygotes at baseline, and at follow-up of 1 and 2 years, and many of these results remained significant after correction for multiple testing (Table 2). We also found that CRP, C3, and CFH were consistently associated with the PET scan measure of AD pathology (HCI) in ε4 homozygotes, but not in participants with other *APOE* genotypes (Figure 2). Further analysis of these proteins by brain region revealed that plasma CRP, C3, and CFH were predominantly negatively associated with FDG score in the default mode network (DMN) of the right brain only among ε4 homozygotes (Figure 3, Table S2 in supporting information). These proteins were not associated with FDG score

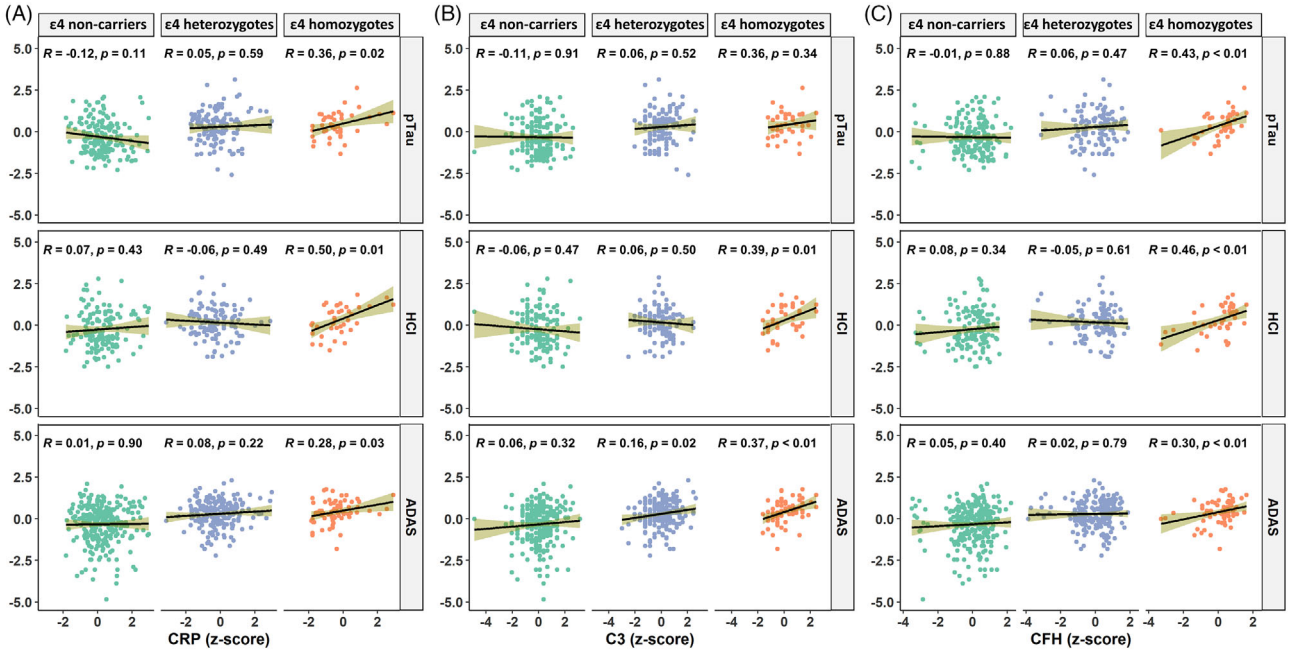


FIGURE 2 The associations between plasma proteins and the AD biomarkers after stratification with *APOE* ε4 genotype. ADNI participants were divided into three groups based on the number of *APOE* ε4 alleles: non-carriers (ε4 = 0), heterozygotes (ε4 = 1), and homozygotes (ε4 = 2). The relationships among three plasma proteins, CRP (A), C3 (B), and CFH (C), and the AD biomarkers, for example, CSF p-tau, the HCI, and (ADAS, at baseline were examined. Scatterplots with a linear regression line with 95% confidence bands (the shaded area), the Pearson coefficient of correlations and their *P*-values were used to illustrate a dose-dependent relationships between a plasma protein (X-axis) and an AD biomarker (Y-axis). AD, Alzheimer's disease; ADAS, Alzheimer's Disease Assessment Scale-Cognitive Subscale; ADNI, Alzheimer's Disease Neuroimaging Initiative; *APOE*, apolipoprotein E; C3, complement C3; CFH, complement factor H; CRP, C-reactive protein; CSF, cerebrospinal fluid; HCI, hypometabolic convergence index; p-tau, phosphorylated tau; WGCNA, weighted gene co-expression network analysis.

in any brain regions among participants with other *APOE* genotypes (Table S2).

4.3 | Further characterizations of plasma CRP, C3, and CFH for AD

We conducted violin-boxplots to compare the levels of these plasma proteins across different status. CRP level was inversely associated with ε4 in a dose-dependent manner ($P = 6.4 \times 10^{-12}$), but expression of C3 and CFH were not different across *APOE* genotype groups (Figure 4A). Across different disease status, plasma CRP level was the lowest, but the levels of C3 and CFH were the highest, in AD compared to MCI and normal controls (Figure 4B); the trend was more so if an AD subject was an ε4 carrier (Figure 4C). CSF Aβ42 level was also inversely associated with the dose of ε4 ($P < 0.0001$, Table 1), but was not impacted by CRP, C3, and CFH regardless of *APOE* genotype (data not shown). Several other proteins in the Green module were modestly associated with ADAS score among ε4 heterozygotes (Table S3 in supporting information).

Consistent with the findings in the ADNI dataset, baseline CRP and CFH levels were associated with AD risk in the FHS dataset (Table 3). Among the other proteins in the Green module, VKDP level was negatively associated with AD risk in ε4 non-carriers but positively associated with AD risk in ε4 carriers in the FHS study (Table S4 in supporting information).

4.4 | Association of proteins in the Black module with AD biomarkers and brain glucose metabolism are *APOE* genotype dependent

Consistent with the finding that the Black module was associated with the AD-related traits among ε4 non-carriers (Figure 1C), levels of several proteins within this module (BNP, Eotaxin-3, and INSLGFBP) were each modestly associated with one AD-related trait among ε4 non-carriers (Table S5 in supporting information).

4.5 | System biology characterization of *APOE* ε4–specific pathways

Enrichment analysis using the five-module data revealed that the most significantly enriched pathways could be grouped into five categories: (1) inflammatory and infectious responses, (2) the complement system, (3) insulin-related metabolism, (4) cell–cell signaling, and (5) cholesterol transport and lipoprotein (Figure 5A). Analysis

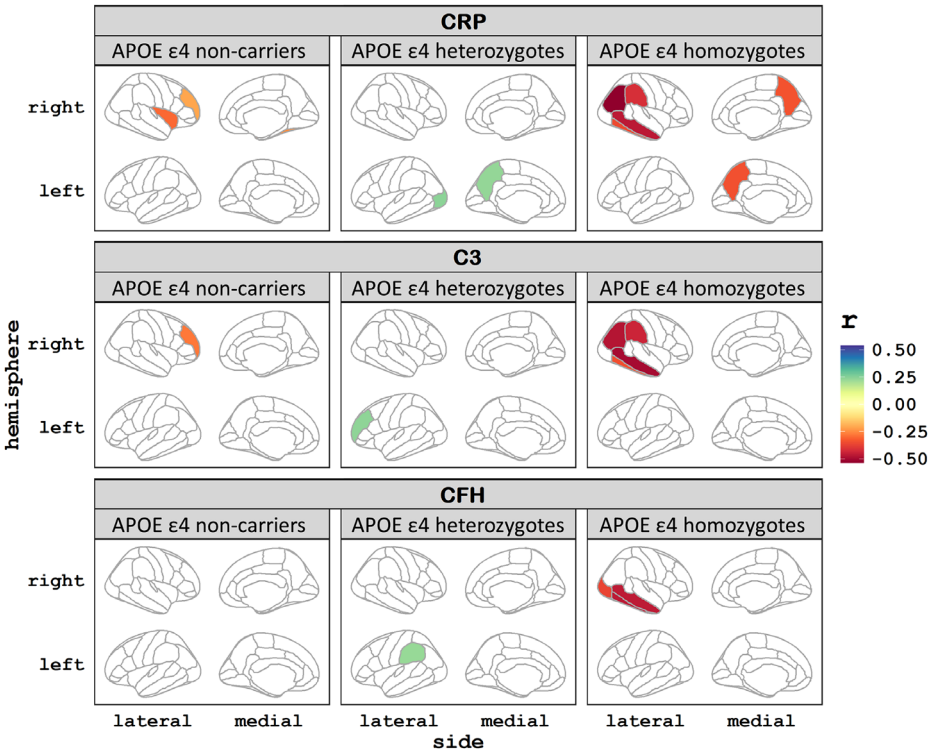


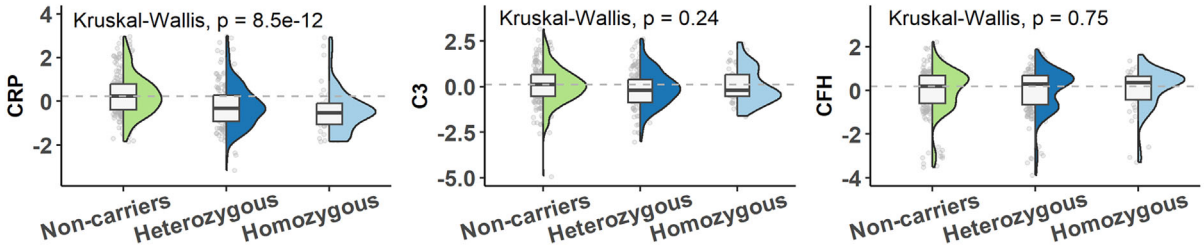
FIGURE 3 The association between plasma proteins and brain glucose metabolism after stratification with *APOE* ε4 genotype. ADNI participants were divided into three groups based on *APOE* ε4 allele: non-carriers (ε4 = 0), heterozygotes (ε4 = 1), and homozygotes (ε4 = 2). Associations of plasma CRP, C3, and CFH with FDG PET scores at different brain regions were analyzed across three *APOE* groups. The FDG PET brain region variables were globally normalized cerebral glucose metabolism (CMRgl). All models were partial correlations between each of the three plasma proteins (CRP, C3, CFH) and CMRgl with adjusted sex and age. Only the brain regions with *P* values < 0.05 were shown. We found that all plasma CRP, C3, and CFH proteins were negatively associated with FDG scores mainly in the right (R) default mode network (DMN) only in *APOE* ε4 homozygotes. Specifically, the brain regions included right (R) parietal inferior cortex, middle and inferior temporal cortex, R temporal pole, R supramarginal cortex, R fusiform, R insula, R and left (L) precuneus, and L cingulum (also refer to Table S2 in supporting information; for the detailed information with the Bonferroni correction). ADAS, Alzheimer's Disease Assessment Scale-Cognitive Subscale; ADNI, Alzheimer's Disease Neuroimaging Initiative; *APOE*, apolipoprotein E; C3, complement C3; CFH, complement factor H; CRP, C-reactive protein; CSF, cerebrospinal fluid; FDG, fluorodeoxyglucose; PET, positron emission tomography.

of the interactions among proteins in the Green module using the STRING database showed that CRP, C3, and CFH interacted with proinflammatory proteins, especially those related to components of the immune response, and apolipoproteins related to lipid metabolism and transport (Figure 5B). Among proteins related to lipid metabolism, apoE and APOA1 were core proteins in the Brown and Black modules, respectively. Apolipoproteins accounted for one half of the members interacting with the Green module (i.e., apoE, APOA2, APOB, and APOC3 and APOA1; Figure 5B, Figure S2). Functional network analysis of all proteins in the five modules also showed that Green module proteins were linked to proteins in the other modules and processes of inflammatory response, blood microparticles, and complement and coagulation cascades (Figure S3 in supporting information). Proteins in the Green module were associated with several other disorders, including age-related macular degeneration (AMD, false discovery rate [FDR] = 0.03), hypertension (FDR = 0.02), immune system diseases (FDR = 0.003), and metabolic diseases (FDR = 0.01).

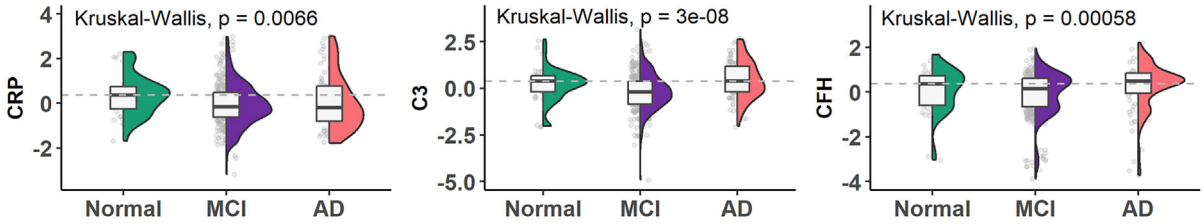
5 | DISCUSSION

Using unbiased data mining and analysis, we found that *APOE* ε4–specific AD risk is modified by the circulating proinflammatory pathway/cascade involving CRP, C3, and CFH (Tables 2 and 3, Figure 2). This pathway has been reported such that CFH binds to monomeric CRP (mCRP) [22–24] and C3,[25] and CFH suppresses CRP and C3 in inflammatory processes.[23,26] CFH also has been implicated in macrophage infiltration, cytokine production, and angiogenesis through its binding to oxidative phospholipids.[27] CRP requires CFH to bind to apoptotic and damaged tissue to activate the complement system[28] and plasma CFH inhibits angiogenesis by reduction of endothelial cell migration.[29] Our findings are consistent with a prior observation that the CFH Y202H polymorphism increases AD risk in ε4 carriers.[30] Previous studies suggest that measures of C3 and CFH in CSF[31] and CFH in blood[32–34] can aid in the differential diagnosis of AD from normal cognition and MCI. In addition, AD brains have increased C3 levels,[35,36] and the C3

(A) All subjects, grouped by APOE ε4 carriers' status

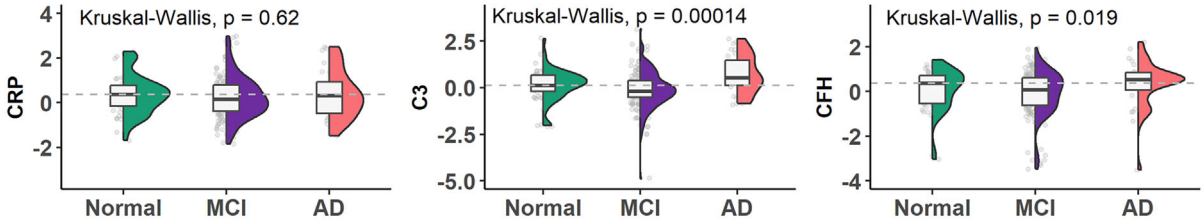


(B) All subjects, grouped by diagnosis

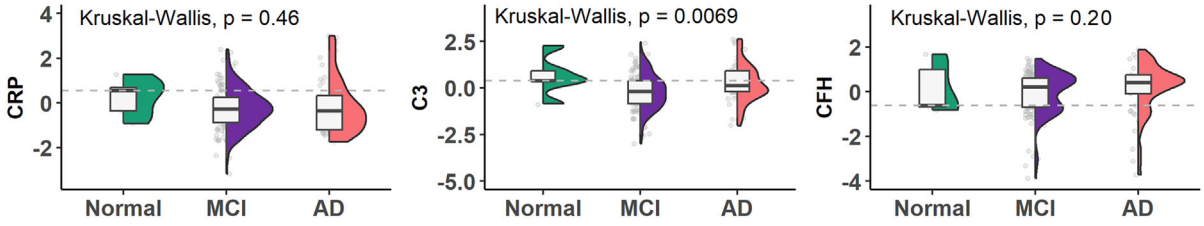


(C) Strata by APOE ε4 carriers' status

APOE ε4 non-carriers



APOE ε4 heterozygous



APOE ε4 homozygous

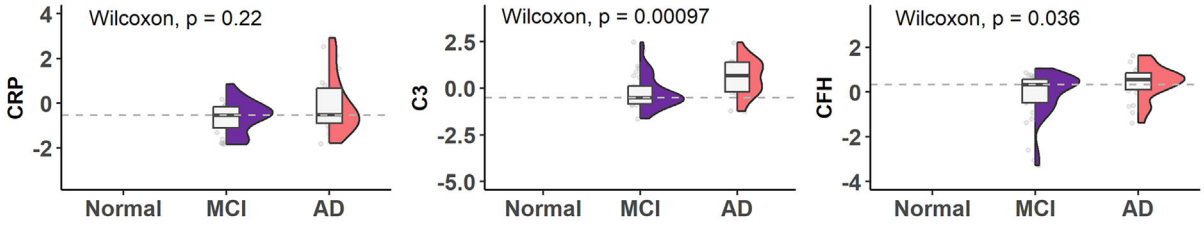


TABLE 3 The associations among three plasma proteins, CRP, C3, or CHF, at baseline and AD risk after stratified by *APOE* ε4 status in the FHS study.

	APOE ε4 non-carriers			*APOE* ε4 carriers		
Proteins	***n***	**HR [95% CI]**	***P* value**	***n***	**HR [95% CI]**	***P* value**
CRP	1261	1.17 [0.93, 1.46]	0.18	374	1.38 [1.02, 1.89]	**0.039**
C3	1261	0.93 [0.73, 1.19]	0.58	374	1.15 [0.85, 1.56]	0.37
CFH	1261	0.95 [0.74, 1.20]	0.64	374	1.41 [1.03, 1.94]	**0.031**

Note: The FHS Offspring (Gen 2) participants were divided into *APOE* ε4 non-carriers and *APOE* ε4 carriers. We used Cox regression models in each *APOE* genotype group to examine the relationship among the three plasma inflammation proteins, CRP, C3, or CFH, and the incidence of AD after adjusting for sex, age, and education. Specifically, we used the *z* score of the log10-transformed values of the protein levels as predictors. We reported the HRs with their 95% CIs. All statistical models were tested using a two-sided hypothesis test with a significance level of $P < 0.05$.

Abbreviations: AD, Alzheimer's disease; *APOE*, apolipoprotein E; C3, complement C3; CFH, complement factor H; CI, confidence interval; CRP, C-reactive protein; FHS, Framingham Heart Study; HR, hazard ratio.

receptor in brain endothelia is associated with neuroinflammation in the brain.[37] These proteins have been shown to be related to other various health issues, including vascular pathology, immune system diseases, and metabolic diseases, most notably AMD.[38–40] Interestingly, AMD risk is associated with *APOE*, but the effect of the ε2 and ε4 alleles is opposite that of AD (i.e., in AMD, ε2 is associated with increased risk whereas ε4 is protective). It is plausible that *APOE* is involved in the response to peripheral infection/inflammation, but the mechanisms of action differ in the eye and brain leading to different pathologies.

While the levels of plasma C3 and CFH were higher in AD than MCI and normal controls, the level of plasma CRP was lower in *APOE* ε4 carriers compared to non-carriers and was lower in AD compared to MCI and normal controls (Figure 4). Although the CRP data seemed paradox, our preclinical study demonstrated that CRP, especially mCRP, penetrates significantly into brain and probably into other tissues as well, leading to a low level of CRP in blood, in *APOE* ε4 mice, but not in *APOE* ε3 and *APOE* ε2 mice.[3] The apoE2 isoform binds to endothelial CD31 and protects brain endothelia from damage caused by the proinflammatory factor mCRP, whereas apoE4 poorly binds to CD31 in the brain, thus allowing mCRP proteins to bind endothelial CD31 more effectively and cause cerebrovascular damage during peripheral inflammation leading to AD pathology.[3] After mCRP is peripherally injected into these mice, *APOE* ε4, but not ε2 and ε3, mice show significantly increased p-tau and neuroinflammation in the brain.[3,41] It has been shown that apoE attenuates peripheral inflammation[42] and modulates inflammatory response in an *APOE* genotype–dependent manner.[43] apoE forms complexes with C1q and regulates C3 activities in the complement cascade, and all three of these proteins colocalize with amyloid plaques in brain.[42,44] Because apoE binds to CFH in blood[45] and adding apoE to cultured monocytes and macrophages enhances CFH's anti-proinflammatory activity,[46] it is likely that *APOE* ε4 binds to CFH to suppress CFH anti-proinflammatory ability leading to brain cerebrovasculature damage, neuroinflammation, and AD.[47] A genetically engineered mouse model with only liver (but no brain) expression of *APOE* ε4 enhances the expression of complement pathways, cognitive impairment, and cerebrovascular damage,[48] indicating peripheral apoE4 protein plays a key role in AD pathogenesis in the brain.

We found that CRP, C3, and CFH were associated with brain glucose hypometabolism assessed by FDG in the regions most vulnerable to AD pathology only in *APOE* ε4 homozygotes (Figure 3). Systemic inflammation and post-viral infections have been shown to be associated with brain glucose hypometabolism.[49–52] In a mouse model of peripheral inflammation and cerebrovascular damage the brain had low FDG scores,[53] whereas Pang et al. demonstrated that apoE mimetic peptides were protective against endothelial damage–increased brain FDG in an animal model of induced subarachnoid hemorrhage.[54] Peripheral insulin resistance has been consistently associated with brain glucose hypometabolism.[55] It is possible that *APOE* genotypes affect brain glucose metabolism through differently modulating peripheral inflammation for brain endothelial dysfunction.

Based on our data, we developed a working hypothesis regarding the contribution of proteins in this pathway to AD and AMD (Figure 5C), taking into account evidence that key members of this pathway (CRP, C3, and CFH) are linked (Figures 1 and 5B, Figure S2). We propose that apoE4 promotes CRP penetration into the cerebrovasculature so that low blood level of CRP is an early step; and then it is followed by increasing blood levels of C3 and CFH in the ε4-specific cascade for AD pathogenesis. Thus, the ε4-specific drug development on CRP for AD should target blocking mCRP binding to

FIGURE 4 Comparison of the blood protein levels across *APOE* genotypes and different diagnoses. All variables (CRP, C3, CFH) underwent a log10 transformation, followed by *z* score rescaling to reduce skewness. After different stratifications, violin boxplots were used to depict the distribution of three blood proteins and the concentrations were compared by using Kruskal–Wallis test. (A) The comparisons were conducted among *APOE* ε4 non-carriers, ε4 heterozygous, and ε4 homozygous carriers. (B) The protein levels were compared across three diagnosis groups: cognitive normal control (normal), MCI, and AD. (C) The participants were first stratified based on *APOE* ε4 carriers' status and then further by the diagnoses. The comparisons across the diagnosis groups in each *APOE* genotype were conducted. The *P* values for statistical significance are shown. AD, Alzheimer's disease; *APOE*, apolipoprotein E; C3, complement C3; CFH, complement factor H; CRP, C-reactive protein; MCI, mild cognitive impairment.

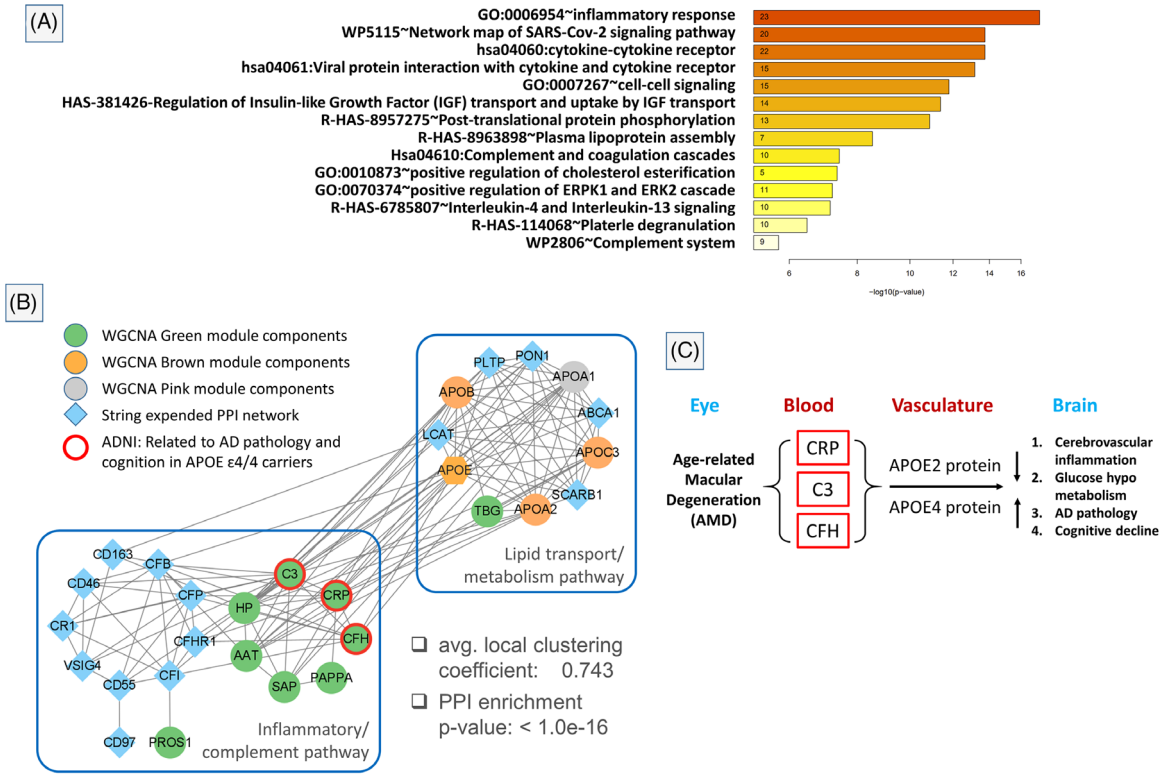


FIGURE 5 System biology characterization of *APOE* ε4–specific pathways. (A) The top pathways of a functional enrichment analysis for the 37 proteins in the five WGCNA modules (Green, Black, Turquoise, Gray, and Brown) are depicted. The numbers of proteins involved in each pathway are illustrated within each bar. (B) The proteins in the Green module were further analyzed by using the PPI network analyses by using STRING program. The extended interactions with CRP, C3, and CFH in PPI were found for the interactions with two groups of proteins outside of the Green module: (1) other proinflammatory proteins and (2) lipid metabolism proteins including *APOE* and other lipid proteins in the Brown module. (C) This study suggested a novel pathological pathway for AD risk in *APOE* ε4 genotype. During peripheral inflammation, an activated cascade, including circulating CRP, C3, and CFH, responds to external or internal infection/injury. In cerebrovasculature, this process is enhanced by *APOE* ε4, but suppressed by *APOE* ε2, leading to AD pathogenesis in the brain; in the eye, this circulating cascade affects the pathological process leading to AMD. AD, Alzheimer's disease; AMD, age-related macular degeneration; *APOE*, apolipoprotein E; C3, complement C3; CFH, complement factor H; CRP, C-reactive protein; PPI, protein–protein interaction; WGCNA, weighted gene co-expression network analysis.

cerebrovasculature, while the drug targets on C3 and CFH can directly lower the blood levels.

Our study has several notable limitations. The C3 association finding was not replicated in the FHS cohort, despite several studies showing an association of blood CRP level and ε4-specific AD risk[6,56,57] and our finding that CFH level was associated with AD among *APOE* ε4 carriers in the ADNI and FHS datasets. This study was observational with the existing data, and did not have an experimental approach to investigate the mechanism of an ε4-specific CRP–C3–CFH pathway. Future studies of independent cohorts and animal models are needed to replicate and prove the findings on the ε4-specific CRP–C3–CFH pathway for AD risk. In addition, there may be other ε4-specific pathways for AD involving the proteins that were not evaluated in this study.

6 | CONCLUSION

In summary, our study of protein biomarkers in blood identified the CRP–C3–CFH inflammation pathway as impacting AD risk in *APOE* ε4 carriers and may serve as potential biomarkers for *APOE* ε4–specific AD diagnosis and prognosis. Ultimately, older people who carry *APOE* ε4 genotype and frequently suffer from systematic infection and inflammation may benefit from new drugs or repurposed existing drugs that targets CRP, C3, and CFH levels to reduce AD risk.

AUTHOR CONTRIBUTIONS

Lindsay A. Farrer and Rhoda Au guided the main mission for the U19 project, and this study was part of it. Wei Qiao Qiu, Qiushan Tao, and Lindsay A. Farrer designed the study and supervised the data analyses. Qiushan Tao and Ting Fang Alvin Ang had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Qiushan Tao conducted the main data analyses for the manuscript. Qiushan Tao and Wei Qiao Qiu drafted the initial manuscript; Lindsay A. Farrer was actively involved in the manuscript writing. Chao Zhang, Gustavo Mercier, Ting Fang Alvin Ang, Samia Akhter-Khan, Zhengrong Zhang, Andrew Taylor, Ron Killiany, Michael Alosco, Jesse Mez, Rhoda Au, and Xiaoling Zhang were

critically involved in the data analyses, the manuscript writing, and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests. The sponsor institutes did not play any role in design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication. Author disclosures are available in the supporting information.

CONSENT STATEMENT

All human subjects provided informed consent for both the ADNI data and Framingham Heart Study. However, for this study, deidentified data was used, ensuring that additional consent was not necessary as the data used was anonymized.

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SUPPORTING INFORMATION

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

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