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APOE4 Copy Number-Dependent Proteomic Changes in the Cerebrospinal Fluid

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Abstract.
Background: *APOE4* has been hypothesized to increase Alzheimer's disease risk by increasing neuroinflammation, though the specific neuroinflammatory pathways involved are unclear.
Objective: Characterize cerebrospinal fluid (CSF) proteomic changes related to *APOE4* copy number.
Methods: We analyzed targeted proteomic data from ADNI CSF samples using a linear regression model adjusting for age, sex, and *APOE4* copy number, and additional linear models also adjusting for AD clinical status or for CSF Aβ, tau, or p-tau levels. False discovery rate was used to correct for multiple comparisons correction.
Results: Increasing *APOE4* copy number was associated with a significant decrease in a CRP peptide level across all five models ($q < 0.05$ for each), and with significant increases in ALDOA, CH3L1 (YKL-40), and FABPH peptide levels ($q < 0.05$ for each) except when controlling for AD clinical status or neurodegeneration biomarkers (i.e., CSF tau or p-tau). In all models except the one controlling for CSF Aβ levels, though not statistically significant, there was a consistent inverse direction of association between *APOE4* copy number and the levels of all 24 peptides from all 8 different complement proteins measured. The odds of this happening by chance for 24 unrelated peptides would be less than 1 in 16 million.
Conclusion: Increasing *APOE4* copy number was associated with decreased CSF CRP levels across all models, and increased CSF ALDOA, CH3L1, and FABH levels when controlling for CSF Aβ levels. Increased *APOE4* copy number may also be associated with decreased CSF complement pathway protein levels, a hypothesis for investigation in future studies.

Keywords: Alzheimer disease, apolipoprotein E4, biomarker, C-reactive protein, cerebrospinal fluid, complement activation, mass spectrometry, neurogenic inflammation

[1]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 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

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INTRODUCTION

The best described genetic contributor to late onset Alzheimer's disease (AD) is the ε4 polymorphism of the apolipoprotein E gene [1]. Individuals carrying a single *APOE4* allele copy have a ∼3-fold increased risk of developing AD, and those who carry two *APOE4* alleles have a greater than 10-fold risk of developing AD [2–5]. Additionally, the presence of an *APOE4* allele is associated with worse neurologic outcomes including a higher index of disability in multiple sclerosis patients, worse cognitive outcomes following mild traumatic brain injury, and increased risk of death following subarachnoid hemorrhage [6–8], as well as increased atherosclerotic cardiovascular disease risk [9]. Likely due to these pleiotropic effects, *APOE4* carriers live ∼4.2 years less than non-*APOE4* carriers [10, 11]. Despite our knowledge of these multiple negative effects of *APOE4*, it remains unclear what the mechanisms are that explain how *APOE4* contributes to AD risk and worse outcomes across these other disease states.

The ApoE protein has multiple biological roles, including cholesterol transport in the central nervous system (CNS), signaling through cell surface receptors, and modulating synaptic function by regulating the expression of syntaxin-1, PSD95, and NMDA and AMPA receptors [12]. Given this multitude of functions, it is unclear which mechanisms explain the increased AD risk in *APOE4* carriers. Patients with an *APOE4* allele are typically diagnosed with AD in their 7th or 8th decade of life, even though the ApoE protein is expressed within the CNS throughout life [13]. This suggests that *APOE4* likely contributes to AD risk before cognitive deficits first appear [14, 15]. This idea is supported by fMRI studies demonstrating that young adult *APOE4* carriers without AD have significant alterations in the default mode network when compared to non-carrier controls [16]. Additionally, young adult *APOE4* carriers show increased activation of the bilateral medial temporal lobe during an encoding task [17], which may be a compensatory mechanism to achieve normal cognitive function in *APOE4* carriers.

One mechanism hypothesized to underlie the link between *APOE4* and AD is neuroinflammation. Indeed, neuroinflammation is a key contributor to AD pathogenesis in humans [18]. Recent evidence in murine models has shown that human *APOE4* knock-in mice have increased glial activation in response to intracerebroventricular LPS injection and increased IL-1β, IL-6, and TNFα levels when compared to *APOE2* or *APOE3* allele knock-in mice [19]. Furthermore, microglia isolated from *APOE4/4* targeted replacement mice, as compared to those from *APOE3/3* mice, have increased pro-inflammatory cytokines such as IL-6, TNFα, and IL12p40 [20, 21]. Although these studies have linked *APOE4* to increases in multiple inflammatory cytokines and pathways, it is unclear which of these inflammatory mechanisms are responsible for increased AD risk in *APOE4* carriers.

Because neurodegeneration in AD itself is associated with inflammation, it is important to study the effect of *APOE4* on the CNS of older adults who do not yet have dementia and neurodegeneration due to AD. Such studies provide an opportunity to discover how *APOE4* affects the CNS and how it increases AD risk, before frank AD-related neurodegeneration begins. Thus, here we analyzed targeted cerebrospinal fluid (CSF) proteomic data from Alzheimer's Disease Neuroimaging Institute (ADNI) research subjects, while controlling for AD clinical status, in order to find CSF protein level variation associated with *APOE4* allele copy number.

METHODS

ADNI study and participants

The patient data and clinical annotations used in this study were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://adni.loni.usc.edu). ADNI is a longitudinal multicenter study that tracks and evaluates changes in cognition, brain structure and function, and biomarkers associated with the progression of mild cognitive impairment (MCI) and AD [22]. Further detail on ADNI is found in the Acknowledgments section. Each ADNI site received written informed consent from all participants and institutional review board approval.

Inclusion and exclusion criteria for the normal control (NC), MCI, and AD cohorts is available at http://adni.loni.usc.edu. Briefly, NC subjects were defined as having a Mini-Mental State Examination (MMSE) [23] score ≥24 and Clinical Dementia Rating (CDR) [24] score of 0 and having no confounding neurological or psychological disorders. MCI subjects had MMSE scores of 23–30, a CDR score of 0.5, objective memory loss as measured by Wechsler Memory Scale Revised—Logical Memory II [25], and preserved activities of daily living. AD patients met the National Institute of Neurological

Table 1
Baseline characteristics of ADNI Patients, grouped by number of *APOE4* alleles Values represent means (SD), or percentages in the case of gender (for females), or count per group (for AT classification)

	0 *APOE4* alleles ($N = 148$)	1 *APOE4* allele ($N = 104$)	2 *APOE4* alleles ($N = 35$)	p
Age	75.94 (6.88)	75.43 (6.77)	71.86 (6.88)	0.007[1]
Gender (Male)	89 (60.1%)	63 (60.6%)	20 (57.1%)	0.935[2]
Race				0.473[3]
Asian	3 (2.0%)	0 (0.0%)	0 (0.0%)	
Black/African American	5 (3.4%)	5 (4.8%)	0 (0.0%)	
White	140 (94.6%)	99 (95.2%)	35 (100.0%)	
Years of Education	16 [14, 18]	16 [14, 18]	16 [14, 16]	0.227[4]
CSF Aβ*	988.47 (397.74)	643.16 (208.95)	482.48 (160.98)	<0.001[1]
CSF Tau**	273.08 (115.89)	335.57 (109.98)	348.32 (120.79)	<0.001[1]
CSF p-tau**	25.72 (12.59)	33.81 (12.73)	35.64 (15.21)	<0.001[1]
Clinical Status				<0.001[2]
Normal	65 (43.9%)	19 (18.3%)	2 (5.7%)	
MCI	64 (43.2%)	53 (51.0%)	18 (51.4%)	
AD	19 (12.8%)	32 (30.8%)	15 (42.9%)	
(ATN) Classification**,+				<0.001[3]
A-T-	59 (40.4%)	8 (7.8%)	0 (0.0%)	
A+T-	37 (25.3%)	26 (25.5%)	9 (25.7%)	
A-T+	13 (8.9%)	3 (2.9 %)	0 (0.0%)	
A+T+	37 (25.3%)	65 (63.7%)	26 (74.3%)	

p-value key: [1]ANOVA, [2]Chi-square, [3]Fisher's Exact, [4]Kruskal Wallis. *28 patients not included who returned values >1700; 4 patients with no BL CSF measures. **4 patients with no BL CSF measures. +$A+$ defined as Aβ values below 1065 pg/ml, $T+$ defined as p-tau values over 27 pg/ml. N classification was not given, because it cannot be determined based on CSF biomarker levels.

and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [26] criteria for probable AD and had MMSE scores of 20–26 and CDR scores of 0.5–1.0. The current study includes CSF samples from 289 unique ADNI-1 subjects (85 normal control, 134 MCI, and 66 AD patients). Publicly available metadata such as age, gender, diagnosis at baseline, MMSE score, and *APOE4* genotype were collected from the ADNI database. Cohort demographics are summarized in Table 1.

ADNI-1 CSF Collection and processing

CSF samples (0.5 mL) were obtained at ADNI visits, stored, transported, and processed according to published procedures [27, 28]. Technical details on the mass spectrometry platform data acquisition, quality control metrics, and validation protocols used in this study are described in the ADNI "Use of Targeted Multiplex Proteomic Strategies to Identify Novel CSF Biomarkers in AD" data primer and in [29]. Briefly, CSF samples were depleted of high abundance proteins using MARS-14 immunoaffinity resin, trypsin digested (1:10 protease:protein ratio), lyophilized, and desalted prior to LC/MRM-MS proteomic analysis on a QTRAP 5500 LC-MS/MS system. CSF multiplex multiple reaction monitoring (MRM) is a standardized peptide panel developed as a QC metric to verify the reproducibility of sample processing and mass spectrometry analysis [30]. A total of 320 peptides produced by tryptic digestion of 143 proteins were identified and met the QC criteria of the ADNI working group for inclusion in the original dataset [29]. These peptides were selected to measure the levels of proteins previously implicated in AD neuropathology and/or neuro-inflammation [29].

Patients in the ADNI proteomics study were classified by AT (i.e., amyloid and p-tau) status, using previously reported CSF Aβ and p-tau measurements made with the Roche Elecsys platform, and previously described Aβ, and p-tau thresholds [31, 32]. We used the AT schema rather than the full ATN classification, because CSF tau and p-tau levels are highly co-linear, such that every patient who would be $T+$ would also be $N+$ and *vice versa* (Shaw LM, personal communication, 6/16/2020).

Statistical analysis

Mass spectrometry data from the ADNI study was re-analyzed to compare peptide data by *APOE4* allele count. Peptides with an expression value below zero were set to missing values. The intraclass correlation (ICC) across technical replicates was calculated for each peptide. Subsequent analysis included 294 peptides that had an ICC ≥ 0.6. The technical replicate for each individual with the smallest number of

missing peptides was used in the analysis. We analyzed CSF targeted proteomic data from 289 research participants in the ADNI-1 study, 85 of whom were healthy controls, 134 of whom had MCI, and 66 of whom had dementia due to AD. Association between each of the variables of interest with each peptide was tested in a linear model framework with an empirical Bayes method for parameter estimation from the limma [33] Bioconductor [34] package. Age and gender were included as cofactors in all models; AD clinical status (i.e., normal, MCI, or dementia due to AD) was also included in model 2. Models 3, 4, and 5 also included either CSF tau or p-tau, or Aβ levels, respectively. False discovery rate was used to correct for multiple hypothesis testing within each statistical model.

RESULTS

ADNI patient cohort characteristics

Baseline characteristics of the ADNI-1 patients whose samples were used for targeted proteomics measurements [29] are presented in Table 1. Subjects with 0, 1, or 2 copies of the *APOE4* allele were similar in terms of gender, race, and years of education. Consistent with prior work showing that the *APOE4* allele is associated with reduced longevity [11], individuals with two *APOE4* allele copies were ∼4 years younger those with zero or one copy of the *APOE4* allele. As expected, the percentage of patients with MCI and AD increased among patients with either 1 or 2 *APOE4* alleles. Consistent with prior work [35, 36], increasing *APOE4* copy number was associated with lower CSF Aβ levels. Increasing *APOE4* copy number was also associated with increases in CSF tau and p-tau levels (Table 1). Lastly, increasing *APOE4* copy number was associated with decreases in the proportion of patients who were A^-T^- and increases in the proportion who were $A+T^+$ (Table 1).

Model 1: CSF proteomic changes and APOE4 gene dosage

To identify protein-derived peptides whose level(s) differed as a function of *APOE4* copy number, a linear model controlling for age and gender was used to test the relationship between *APOE4* copy number and CSF peptide levels. Initial analysis evaluated 294 peptides with sufficient replicability (ICC $\geq$ 0.6) for measuring CSF expression variance by *APOE4* copy number (Table 2). In this model, 12

Table 2
Proteins included in the ADNI Targeted CSF Proteomics Study

1433Z	CMGA	IFNB	NELL2	SCG3
A1AT	CNDP1	IGSF8	NEO1	SDCB1
A1AT	CNTF	IL10	NEUS	SE6L1
A1BG	CNTN1	IL12B	NFH	SHSA7
A2GL	CNTN2	IL17	NFL	SIAE
A2MG	CO2	IL1A	NFM	SLIK1
A4	CO3	IL27A	NGF	SMOC1
AACT	CO4A	IL6	NICA	SODC
AATM	CO5	IL6RA	NLGN3	SODE
AFAM	CO6	ITIH1	NPTX1	SORC1
ALDOA	CO8B	ITIH5	NPTX2	SORC2
AMBP	COCH	ITM2B	NPTXR	SORC3
AMD	CRP	JAK1	NPY	SPON1
APLP2	CSTN1	KAIN	NRCAM	SPRL1
APOA	CSTN3	KCC2B	NRX1A	STX12
APOA1	CUTA	KI67	NRX2A	SV2A
APOB	CYTC	KLK10	NRX3A	SYNJ1
APOC1	DAG1	KLK11	NSG1	SYT11
APOD	DIAC	KLK12	OSTP	TADBP
APOE	ENOG	KLK3	PCD17	TAU
B2MG	ENPP2	KLK6	PCMD1	TCRG1
B3GN1	EXTL2	KLK9	PCSK1	TEN3
BACE1	FABP5	KLKB1	PDIA3	TGFB1
BASP1	FABP6	KNG1	PDYN	TGFB2
BDNF	FABP7	KPCZ	PEDF	TGFB3
BTD	FABPH	KPYM	PGRP2	TGON2
C1QA	FABPI	L1CAM	PIMT	THRB
C1QB	FAM3C	LAMB2	PLDX1	TIMP1
C3AR	FBLN1	LFTY2	PLMN	TNF14
CA2D1	FBLN3	LPHN1	PPN	TNFA
CAD13	FETUA	LRC4B	PRDX1	TNR1B
CADM3	FMOD	LTBP2	PRDX2	TNR21
CAH1	GFAP	MIME	PRDX3	TNR6
CATA	GLNA	MMP2	PRDX4	TRBM
CATD	GOGB1	MMP9	PRDX5	TRFE
CATL1	GOLM1	MMRN2	PRDX6	TRFM
CCKN	GRIA4	MOG	PTGDS	TTHY
CCL25	HBA	MTHR	PTPRD	UBB
CD14	HBB	MUC18	PTPRN	UCHL1
CD59	HEMO	NBL1	PVRL1	VASN
CERU	HERC4	NCAM1	RIMS3	VGF
CFAB	I18BP	NCAM2	SAP	VTDB
CH3L1	IBP2	NCAN	SCG1	X3CL1
CLUS	IBP6	NEGR1	SCG2	

of 294 peptides had significant expression changes ($q \leq 0.05$) associated with increasing *APOE4* copy number (Table 3 and Fig. 1A). CSF levels of an *APOE4*-specific peptide (APOE_LGADMEDVR) were substantially higher in *APOE4* carriers versus non-carriers (q- = 6.53 x 10^{-81}), consistent with prior studies [37, 38]. Two peptides found in all *APOE* isoforms had elevated CSF expression with increasing *APOE4* copy number (APOE_LAVYQAGAR, $q = 0.027$; APOE_LGPLVEQGR, $q = 0.027$). CSF expression of an *APOE2*-specific peptide was found to decrease with higher *APOE4* gene dosage (APOE_CLAVYQAGAR, $q = 0.027$).

Table 3
Expression of the Top 20 Peptides and for all complement peptides/proteins by *APOE4* copy number in multivariate models accounting for age and gender (model 1). For full data set, see Supplementary File 1

Top 20 proteins/peptides whose expression differed as a function of APOE4 copy number, controlling for age, gender, and clinical status (normal, MCI or AD)

Protein	UniProt ID	Peptide	Log Fold Change	Average Expression	*t* statistic	p	FDR Corrected p
APOE	P02649	LGADMEDVR	3.594	9.193	27.105	0.000	0.000
CRP	P02741	ESDTSYVSLK	–0.633	15.228	–4.074	0.000	0.009
APOE	P02649	LGPLVEQGR	0.287	22.419	3.588	0.000	0.038
APOE	P02649	LAVYQAGAR	0.292	25.410	3.421	0.001	0.053
ALDOA	P04075	ALQASALK	0.121	19.168	3.105	0.002	0.077
CH3L1	P36222	ILGQQVPYATK	0.112	23.114	3.133	0.002	0.077
CH3L1	P36222	SFTLASSETGVGAPISGPGIPGR	0.112	18.225	3.107	0.002	0.077
FABPH	P05413	SIVTLDGGK	0.131	14.664	3.208	0.001	0.077
ALDOA	P04075	QLLLTADDR	0.111	16.246	2.963	0.003	0.079
AMBP	P02760	FLYHK	–0.167	11.800	–2.953	0.003	0.079
APOE	P02649	AATVGSLAGQPLQER	0.247	20.145	3.039	0.003	0.079
APOE	P02649	CLAVYQAGAR	–0.630	8.715	–2.919	0.004	0.079
CH3L1	P36222	VTIDSSYDIAK	0.109	21.303	2.915	0.004	0.079
FABPH	P05413	SLGVGFATR	0.108	15.706	2.998	0.003	0.079
KNG1	P01042	TVGSDTFYSFK	–0.185	15.118	–2.898	0.004	0.079
AMBP	P02760	ETLLQDFR	–0.171	18.977	–2.777	0.006	0.107
A2GL	P02750	DLLLPQPDLR	–0.148	25.898	–2.701	0.007	0.127
A2GL	P02750	VAAGAFQGLR	–0.139	23.017	–2.458	0.015	0.152
AATC	P17174	IVASTLSNPELFEEWTGNVK	0.086	12.727	2.517	0.012	0.152
AATM	P00505	FVTVQTISGTGALR	0.097	10.314	2.496	0.013	0.152

CSF Complement proteins/peptides expression as a function of APOE4 copy number, controlling for age, gender, and clinical status (normal, MCI or AD)

Protein	UniProt ID	Peptide	Log Fold Change	Average Expression	*t* statistic	p	FDR Corrected p
C1QB	P02746	LEQGENVFLQATDK	–0.042	17.128	–1.241	0.216	0.622
C1QB	P02746	VPGLYYFTYHASSR	–0.053	17.604	–1.135	0.257	0.669
CFAB	P00751	DAQYAPGYDK	–0.134	16.401	–2.435	0.016	0.152
CFAB	P00751	VSEADSSNADWVTK	–0.111	16.220	–2.471	0.014	0.152
CFAB	P00751	YGLVTYATYPK	–0.134	22.532	–2.474	0.014	0.152
CO2	P06681	DFHINLFR	–0.065	18.691	–1.232	0.219	0.623
CO2	P06681	HAIILLTDGK	–0.070	15.695	–1.625	0.105	0.412
CO2	P06681	SSGQWQTPGATR	–0.077	15.922	–1.689	0.092	0.377
CO3	P01024	IHWESASLLR	–0.267	14.006	–2.136	0.034	0.235
CO3	P01024	TELRPGETLNVNFLLR	–0.081	10.150	–1.997	0.047	0.259
CO4A	P0C0L4	DHAVDLIQK	–0.038	22.142	–0.745	0.457	0.858
CO4A	P0C0L4	GSFEFPVGDAVSK	–0.035	25.297	–0.611	0.541	0.879
CO4A	P0C0L4	LGQYASPTAK	–0.041	21.701	–0.760	0.448	0.857
CO4A	P0C0L4	NVNFQK	–0.019	18.387	–0.392	0.695	0.978
CO4A	P0C0L4	VLSLAQEQVGGSPEK	–0.049	19.989	–1.039	0.300	0.734
CO4A	P0C0L4	VTASDPLDTLGSEGALSPGGVASLLR	–0.029	18.008	–0.712	0.477	0.871
CO5	P01031	DINYVNPVIK	–0.045	16.181	–0.630	0.529	0.876
CO5	P01031	TLLPVSKPEIR	–0.050	17.209	–0.757	0.449	0.857
CO5	P01031	VFQFLEK	–0.043	18.210	–0.644	0.520	0.873
CO6	P13671	ALNHLPLEYNSALYSR	–0.113	16.297	–1.772	0.077	0.345
CO6	P13671	SEYGAALAWEK	–0.118	15.760	–2.072	0.039	0.240
CO8B	P07358	IPGIFELGISSQSDR	–0.057	14.476	–0.761	0.447	0.857
CO8B	P07358	SDLEVAHYK	–0.082	13.090	–1.342	0.181	0.571
CO8B	P07358	YEFILK	–0.077	18.883	–1.263	0.208	0.610

Log Fold Change - log2 fold change of peptide expression with each additional *APOE4* allele copy in multivariate models for all analyzed CSF samples; Average expression – average peptide expression in all analyzed CSF samples; *t* statistic – hypothesis test statistic estimating the mean peptide expression (population mean) from the sampling distribution for each peptide in all analyzed CSF samples.

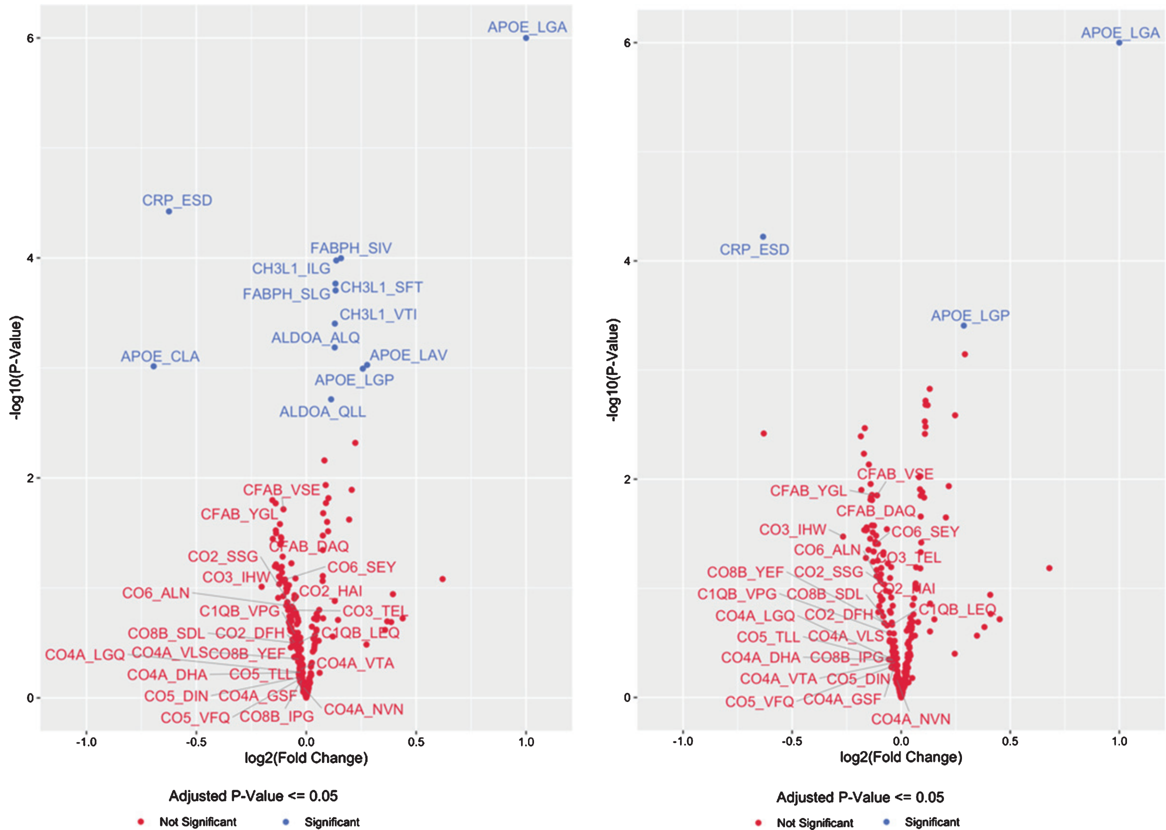

Fig. 1. Volcano Plot of CSF Protein/Peptide Expression by *APOE* genotype, for the top 20 proteins and the complement cascade proteins in model 1 (A), and for the top 20 proteins and the complement cascade proteins in model 2 (B).

Increasing *APOE4* allele copy number was associated with reduced expression of a peptide from the acute inflammatory marker C-reactive protein (CRP) ($q = 0.006$). Increasing *APOE4* copy number was also associated with increasing expression of peptides derived from the glycoprotein Chitinase 3-like protein 1 (CH3L1; also known as YKL-40) ($q < 0.05$), the cardiac injury biomarker heart-type fatty acid binding protein (FABPH) ($q < 0.05$), and the glycolytic enzyme fructose-bisphosphate aldolase A (ALDOA; $q < 0.05$).

There was a consistent inverse direction of association between *APOE4* copy number and CSF levels of all 24 peptides from all 8 complement pathway proteins measured (Table 3, Fig. 1A), although these effects were not significant for any individual complement protein-derived peptide ($p > 0.05$ for each, prior to multiple correction comparison). Nonetheless, the odds of this happening for 24 unrelated peptides by chance would be 1 over 2^{24}, or less than 1 in 16 million. Alternatively, since these 24 peptides were derived from 8 complement pathway proteins, the odds of 8 proteins at random all showing consistently lower expression as a function of *APOE4* allele copy number, even if the change for each individual protein was not statistically significant, would be $1/2^8$, or a 1 out of 256 chance.

Model 2: APOE4-dependent CSF peptide changes and clinical status

Because *APOE4* is found in AD patients at disproportionately high frequencies compared to the general population, it is possible that the above findings reflect confounding by AD clinical status (and neurodegeneration) rather than changes directly related to increased *APOE4* copy number itself. Therefore, a second linear model was used that corrected for clinical status (normal control, MCI, or dementia due to AD) in addition to the items in model 1, to test for associations between *APOE4* copy number and CSF peptide expression levels. In this second model, only 3 of 294 peptides had statistically significant *APOE4* copy

number-related changes in CSF expression levels (Table 4 and Fig. 1B). Increasing *APOE4*-copy number was associated with increased expression of the *APOE4*-specific peptide (LGADMEDVR) ($q < 0.01$) and decreased expression the CRP-derived peptide (ESDTSYVSLK) ($q < 0.01$). A pan-APOE peptide (LGPLVEQGR) had increased expression associated with increased *APOE4* copy number in this model ($q = 0.038$). CH3L1 (YKL-40)-, FABPH-, and ALDOA-derived peptides that showed significant *APOE4*- copy number-related changes in expression in model 1 (above) no longer remained statistically significant after correcting for disease status and multiple comparisons, although there was still a trend toward increased CSF protein expression for each ($q = 0.077$, $q = 0.077$, and $q = 0.079$, respectively). As in the first model (not controlling for AD clinical status), none of the 24 complement protein-derived peptides demonstrated statistically significant differences as a function of *APOE4* copy number. Yet, as in the first model, in this model there was a consistent inverse direction of association between *APOE4* copy number and expression of all 24 peptides from all 8 different complement proteins measured (Table 4). Although none of these inverse associations were statistically significant on their own ($p > 0.05$ for each, prior to multiple comparison) the odds of 24 unrelated peptides all showing this pattern of decreased expression by chance would be less than 1 over 2^{24}, or less than 1 in 16 million. Alternatively, all 24 of these peptides were derived from 8 complement pathway proteins. Thus the odds of 8 proteins all showing lower expression levels as a function of *APOE4* allele copy number, even if the change in each individual protein was not statistically significant at $p < 0.05$, would be $1/2^8$, or a 1 out of 256 chance.

Model 3: APOE4-dependent CSF peptide changes and CSF tau levels

To determine whether *APOE4*-dependent CSF proteomic changes were independent of changes in CSF tau levels (a non-specific indicator of neurodegeneration [39]), we constructed a third model for associations between *APOE4* copy number and CSF peptide expression levels, correcting for CSF tau levels, age, and gender. In this third model, 183 of 294 peptides from 132 proteins had statistically significant *APOE4* copy number-related changes in CSF expression levels (Table 5, Supplementary File 2). Similar to the findings in models 1 and 2, in model 3 increasing *APOE4* copy number was associated with increased expression of the *APOE4*-specific peptide (LGADMEDVR) ($q = 1.5 \times 10^{-75}$) and decreased expression of the CRP-derived peptide (ESDTSYVSLK) ($q = 0.0006$; Supplementary File 2). CH3L1 (YKL-40)-, FABPH-, and ALDOA-derived peptides that showed significant *APOE4* copy number-related changes in expression in model 1 (above) no longer remained statistically significant after correcting for CSF tau levels in model 3 ($q > 0.05$ for each; Supplementary File 2).

Four peptides from the complement cascade proteins C1QB, CFAB and CO2 showed significantly reduced expression as a function of increasing *APOE4* allele count in model 3 ($q < 0.05$ for each; Table 5). There was also a consistently inverse direction of association between *APOE4* copy number and CSF levels for the 20 other peptides from all 8 complement proteins measured (Table 5), although these effects were not statistically significant after multiple comparison correction ($q > 0.05$ for each).

Model 4: APOE4-dependent CSF peptide changes and CSF p-tau levels

To determine whether *APOE4*-dependent CSF proteomic changes were independent of changes in CSF p-tau levels (a more specific indicator of AD-related neurodegeneration [39]), we constructed a fourth model for associations between *APOE4* copy number and CSF peptide expression levels, correcting for CSF p-tau levels, age, and gender. In this fourth model, 184 of 294 peptides from 132 proteins had statistically significant *APOE4* copy number-related changes in CSF expression levels (Table 6). Similar to the findings in models 1 and 2, in model 3 increasing *APOE4*-copy number was associated with increased expression of the *APOE4*-specific peptide (LGADMEDVR) ($q = 4.1 \text{ x } 10^{-74}$) and decreased expression of the CRP-derived peptide (ESDTSYVSLK) ($q = 0.00094$). As in model 3 (controlling for CSF tau levels), CH3L1 (YKL-40)-, FABPH-, and ALDOA-derived peptides that showed significant *APOE4*- copy number-related changes in expression observed in model 1 no longer remained statistically significant after correcting for CSF p-tau levels ($q > 0.05$ for each; Supplementary File 2).

Three peptides from the complement cascade proteins C1QB, CFAB, and CO2 showed significantly reduced expression as a function of increasing *APOE4* allele count ($q < 0.05$ for each) in model 4. There was also a consistently inverse direction of

Table 4
Expression of the Top 20 Peptides and for all complement peptides/proteins by *APOE4* copy number in multivariate models accounting for age and gender and clinical status (Model 2). For full data set, see Supplementary File 1

Top 20 proteins/peptides whose expression differed as a function of APOE4 copy number, controlling for age, gender, and clinical status (normal, MCI or AD)

Protein	UniProt ID	Peptide	Log Fold Change	Average Expression	*t* statistic	p	FDR Corrected p
APOE	P02649	LGADMEDVR	3.594	9.193	27.105	0.000	0.000
CRP	P02741	ESDTSYVSLK	–0.633	15.228	–4.074	0.000	0.009
APOE	P02649	LGPLVEQGR	0.287	22.419	3.588	0.000	0.038
APOE	P02649	LAVYQAGAR	0.292	25.410	3.421	0.001	0.053
ALDOA	P04075	ALQASALK	0.121	19.168	3.105	0.002	0.077
CH3L1	P36222	ILGQQVPYATK	0.112	23.114	3.133	0.002	0.077
CH3L1	P36222	SFTLASSETGVGAPISGPGIPGR	0.112	18.225	3.107	0.002	0.077
FABPH	P05413	SIVTLDGGK	0.131	14.664	3.208	0.001	0.077
ALDOA	P04075	QLLLTADDR	0.111	16.246	2.963	0.003	0.079
AMBP	P02760	FLYHK	–0.167	11.800	–2.953	0.003	0.079
APOE	P02649	AATVGSLAGQPLQER	0.247	20.145	3.039	0.003	0.079
APOE	P02649	CLAVYQAGAR	–0.630	8.715	–2.919	0.004	0.079
CH3L1	P36222	VTIDSSYDIAK	0.109	21.303	2.915	0.004	0.079
FABPH	P05413	SLGVGFATR	0.108	15.706	2.998	0.003	0.079
KNG1	P01042	TVGSDTFYSFK	–0.185	15.118	–2.898	0.004	0.079
AMBP	P02760	ETLLQDFR	–0.171	18.977	–2.777	0.006	0.107
A2GL	P02750	DLLLPQPDLR	–0.148	25.898	–2.701	0.007	0.127
A2GL	P02750	VAAGAFQGLR	–0.139	23.017	–2.458	0.015	0.152
AATC	P17174	IVASTLSNPELFEEWTGNVK	0.086	12.727	2.517	0.012	0.152
AATM	P00505	FVTVQTISGTGALR	0.097	10.314	2.496	0.013	0.152

CSF Complement proteins/peptides expression as a function of APOE4 copy number, controlling for age, gender, and clinical status (normal, MCI or AD)

Protein	UniProt ID	Peptide	Log Fold Change	Average Expression	*t* statistic	p	FDR Corrected p
C1QB	P02746	LEQGENVFLQATDK	–0.042	17.128	–1.241	0.216	0.622
C1QB	P02746	VPGLYYFTYHASSR	–0.053	17.604	–1.135	0.257	0.669
CFAB	P00751	DAQYAPGYDK	–0.134	16.401	–2.435	0.016	0.152
CFAB	P00751	VSEADSSNADWVTK	–0.111	16.220	–2.471	0.014	0.152
CFAB	P00751	YGLVTYATYPK	–0.134	22.532	–2.474	0.014	0.152
CO2	P06681	DFHINLFR	–0.065	18.691	–1.232	0.219	0.623
CO2	P06681	HAIILLTDGK	–0.070	15.695	–1.625	0.105	0.412
CO2	P06681	SSGQWQTPGATR	–0.077	15.922	–1.689	0.092	0.377
CO3	P01024	IHWESASLLR	–0.267	14.006	–2.136	0.034	0.235
CO3	P01024	TELRPGETLNVNFLLR	–0.081	10.150	–1.997	0.047	0.259
CO4A	P0C0L4	DHAVDLIQK	–0.038	22.142	–0.745	0.457	0.858
CO4A	P0C0L4	GSFEFPVGDAVSK	–0.035	25.297	–0.611	0.541	0.879
CO4A	P0C0L4	LGQYASPTAK	–0.041	21.701	–0.760	0.448	0.857
CO4A	P0C0L4	NVNFQK	–0.019	18.387	–0.392	0.695	0.978
CO4A	P0C0L4	VLSLAQEQVGGSPEK	–0.049	19.989	–1.039	0.300	0.734
CO4A	P0C0L4	VTASDPLDTLGSEGALSPGGVASLLR	–0.029	18.008	–0.712	0.477	0.871
CO5	P01031	DINYVNPVIK	–0.045	16.181	–0.630	0.529	0.876
CO5	P01031	TLLPVSKPEIR	–0.050	17.209	–0.757	0.449	0.857
CO5	P01031	VFQFLEK	–0.043	18.210	–0.644	0.520	0.873
CO6	P13671	ALNHLPLEYNSALYSR	–0.113	16.297	–1.772	0.077	0.345
CO6	P13671	SEYGAALAWEK	–0.118	15.760	–2.072	0.039	0.240
CO8B	P07358	IPGIFELGISSQSDR	–0.057	14.476	–0.761	0.447	0.857
CO8B	P07358	SDLEVAHYK	–0.082	13.090	–1.342	0.181	0.571
CO8B	P07358	YEFILK	–0.077	18.883	–1.263	0.208	0.610

Log Fold Change - log2 fold change of peptide expression with each additional *APOE4* allele copy in multivariate models for all analyzed CSF samples; Average expression – average peptide expression in all analyzed CSF samples; *t* statistic – hypothesis test statistic estimating the mean peptide expression (population mean) from the sampling distribution for each peptide in all analyzed CSF samples.

Table 5
Expression for the Top 20 Peptides and for all complement peptides/proteins by *APOE4* copy number in multivariate models accounting for age, gender and CSF tau levels (Model 3). For full data set, see Supplementary File 2

Proteins/peptides whose expression differed as a function of APOE4 copy number, controlling for age, gender, and CSF tau level (in order of significance).

Protein	UniProt ID	Peptide	Log Fold Change	Average Expression	*t* statistic	p	FDR Corrected p
APOE	P02649	LGADMEDVR	3.365	9.208	26.640	0.000	0.000
A4	P05067	LVFFAEDVGSNK	–0.166	18.133	–5.315	0.000	0.000
NCAM2	O15394	IIELSQTTAK	–0.171	19.631	–5.342	0.000	0.000
VGF	O15240	AYQGVAAPFPK	–0.254	17.733	–5.299	0.000	0.000
AMD	P19021	IVQFSPSGK	–0.185	21.247	–4.964	0.000	0.000
CA2D1	P54289	FVVTDGGITR	–0.187	20.267	–4.943	0.000	0.000
CMGA	P10645	YPGPQAEGDSEGLSQGLVDR	–0.237	15.851	–5.008	0.000	0.000
IGSF8	Q969P0	LQGDAVVLK	–0.133	18.301	–4.885	0.000	0.000
IGSF8	Q969P0	VVAGEVQVQR	–0.119	19.050	–4.910	0.000	0.000
NBL1	P41271	LALFPDK	–0.141	26.936	–4.998	0.000	0.000
SCG2	P13521	VLEYLNQEK	–0.202	20.916	–4.897	0.000	0.000
VGF	O15240	NSEPQDEGELFQGVDPR	–0.252	19.459	–4.923	0.000	0.000
VGF	O15240	THLGEALAPLSK	–0.244	16.748	–4.919	0.000	0.000
FAM3C	P84101	SPFEQHIK	–0.159	19.027	–4.815	0.000	0.000
AMD	P19021	IPVDEEAFVIDFKPR	–0.167	17.376	–4.735	0.000	0.000
AMD	P19021	NGQWTLIGR	–0.180	16.249	–4.725	0.000	0.000
CA2D1	P54289	TASGVNQLVDIYEK	–0.171	13.242	–4.712	0.000	0.000
CYTC	P01034	ALDFAVGEYNK	–0.114	34.102	–4.717	0.000	0.000
NPTX2	P47972	TESTLNALLQR	–0.282	10.581	–4.723	0.000	0.000
CAD13	P55290	YEVSSPYFK	–0.161	22.725	–4.657	0.000	0.000

CSF Complement proteins/peptides expression as a function of APOE4 copy number, controlling for age, gender, and tau

Protein	UniProt ID	Peptide	Log Fold Change	Average Expression	*t* statistic	p	FDR Corrected p
C1QB	P02746	LEQGENVFLQATDK	–0.071	17.131	–2.124	0.034	0.055
C1QB	P02746	VPGLYYFTYHASSR	–0.132	17.608	–2.921	0.004	0.008
CFAB	P00751	DAQYAPGYDK	–0.111	16.394	–2.015	0.045	0.068
CFAB	P00751	VSEADSSNADWVTK	–0.055	16.211	–1.274	0.204	0.242
CFAB	P00751	YGLVTYATYPK	–0.124	22.526	–2.270	0.024	0.039
CO2	P06681	DFHINLFR	–0.088	18.688	–1.670	0.096	0.129
CO2	P06681	HAIILLTDGK	–0.101	15.692	–2.360	0.019	0.032
CO2	P06681	SSGQWQTPGATR	–0.102	15.920	–2.228	0.027	0.043
CO3	P01024	IHWESASLLR	–0.254	13.995	–2.022	0.044	0.067
CO3	P01024	TELRPGETLNVNFLLR	–0.083	10.147	–2.028	0.044	0.067
CO4A	P0C0L4	DHAVDLIQK	–0.087	22.140	–1.731	0.085	0.117
CO4A	P0C0L4	GSFEFPVGDAVSK	–0.098	25.295	–1.774	0.077	0.109
CO4A	P0C0L4	LGQYASPTAK	–0.106	21.701	–2.052	0.041	0.064
CO4A	P0C0L4	NVNFQK	–0.071	18.386	–1.490	0.137	0.176
CO4A	P0C0L4	VLSLAQEQVGGSPEK	–0.062	19.985	–1.317	0.189	0.229
CO4A	P0C0L4	VTASDPLDTLGSEGALSPGGVASLLR	–0.067	18.006	–1.659	0.098	0.131
CO5	P01031	DINYVNPVIK	–0.096	16.181	–1.346	0.179	0.220
CO5	P01031	TLLPVSKPEIR	–0.076	17.208	–1.141	0.255	0.293
CO5	P01031	VFQFLEK	–0.070	18.209	–1.053	0.293	0.328
CO6	P13671	ALNHLPLEYNSALYSR	–0.112	16.292	–1.752	0.081	0.113
CO6	P13671	SEYGAALAWEK	–0.104	15.755	–1.817	0.070	0.102
CO8B	P07358	IPGIFELGISSQSDR	–0.045	14.471	–0.595	0.552	0.584
CO8B	P07358	SDLEVAHYK	–0.055	13.083	–0.883	0.378	0.413
CO8B	P07358	YEFILK	–0.045	18.876	–0.727	0.468	0.499

association between *APOE4* copy number and CSF levels for the 21 other peptides from all 8 complement proteins measured (Table 6), although these effects were not statistically significant after multiple comparison correction in model 4 ($q > 0.05$ for each).

Model 5: APOE4-dependent CSF peptide changes and CSF Aβ levels

To determine whether *APOE4*-dependent CSF proteomic changes were independent of changes in CSF Aβ levels, we constructed a fifth model for

Table 6
Expression of the Top 20 Indicated Peptides and for all complement peptides/proteins by *APOE4* copy number in multivariate models accounting for age, gender and CSF p-tau levels (model 4). For full data set, see Supplementary File 2

Proteins/peptides whose expression differed as a function of APOE4 copy number, controlling for age, gender, and ptau

Protein	UniProt ID	Peptide	Log Fold Change	Average Expression	*t* statistic	p	FDR Corrected p
APOE	P02649	LGADMEDVR	3.340	9.208	26.198	0.000	0.000
A4	P05067	LVFFAEDVGSNK	–0.168	18.133	–5.170	0.000	0.000
NCAM2	O15394	IIELSQTTAK	–0.175	19.631	–5.197	0.000	0.000
VGF	O15240	AYQGVAAPFPK	–0.258	17.733	–5.151	0.000	0.000
AMD	P19021	IVQFSPSGK	–0.190	21.247	–4.896	0.000	0.000
CA2D1	P54289	FVVTDGGITR	–0.192	20.267	–4.878	0.000	0.000
CMGA	P10645	YPGPQAEGDSEGLSQGLVDR	–0.245	15.851	–4.931	0.000	0.000
NBL1	P41271	LALFPDK	–0.143	26.936	–4.855	0.000	0.000
VGF	O15240	NSEPQDEGELFQGVDPR	–0.258	19.459	–4.857	0.000	0.000
FAM3C	P84101	SPFEQHIK	–0.165	19.027	–4.768	0.000	0.000
IGSF8	Q969P0	VVAGEVQVQR	–0.121	19.050	–4.784	0.000	0.000
SCG2	P13521	VLEYLNQEK	–0.207	20.916	–4.822	0.000	0.000
VGF	O15240	THLGEALAPLSK	–0.249	16.748	–4.799	0.000	0.000
IGSF8	Q969P0	LQGDAVVLK	–0.135	18.301	–4.735	0.000	0.000
AMD	P19021	IPVDEEAFVIDFKPR	–0.170	17.376	–4.623	0.000	0.000
AMD	P19021	NGQWTLIGR	–0.183	16.249	–4.615	0.000	0.000
CA2D1	P54289	TASGVNQLVDIYEK	–0.175	13.242	–4.641	0.000	0.000
CAD13	P55290	YEVSSPYFK	–0.167	22.725	–4.601	0.000	0.000
CMGA	P10645	SEALAVDGAGKPGAEEAQDPEGK	–0.216	17.989	–4.597	0.000	0.000
CMGA	P10645	SGEATDGARPQALPEPMQESK	–0.206	19.085	–4.642	0.000	0.000

CSF Complement proteins/peptides expression as a function of APOE4 copy number, controlling for age, gender, and ptau

Protein	UniProt ID	Peptide	Log Fold Change	Average Expression	*t* statistic	p	FDR Corrected p
C1QB	P02746	LEQGENVFLQATDK	–0.069	17.131	–2.054	0.041	0.064
C1QB	P02746	VPGLYYFTYHASSR	–0.130	17.608	–2.813	0.005	0.010
CFAB	P00751	DAQYAPGYDK	–0.107	16.394	–1.925	0.055	0.083
CFAB	P00751	VSEADSSNADWVTK	–0.048	16.211	–1.102	0.271	0.319
CFAB	P00751	YGLVTYATYPK	–0.119	22.526	–2.169	0.031	0.049
CO2	P06681	DFHINLFR	–0.086	18.688	–1.610	0.109	0.146
CO2	P06681	HAIILLTDGK	–0.100	15.692	–2.293	0.023	0.038
CO2	P06681	SSGQWQTPGATR	–0.099	15.920	–2.137	0.033	0.053
CO3	P01024	IHWESASLLR	–0.249	13.995	–1.966	0.050	0.077
CO3	P01024	TELRPGETLNVNFLLR	–0.082	10.147	–1.982	0.048	0.074
CO4A	P0C0L4	DHAVDLIQK	–0.080	22.140	–1.563	0.119	0.157
CO4A	P0C0L4	GSFEFPVGDAVSK	–0.091	25.295	–1.596	0.112	0.149
CO4A	P0C0L4	LGQYASPTAK	–0.099	21.701	–1.866	0.063	0.092
CO4A	P0C0L4	NVNFQK	–0.064	18.386	–1.314	0.190	0.234
CO4A	P0C0L4	VLSLAQEQVGGSPEK	–0.053	19.985	–1.114	0.266	0.314
CO4A	P0C0L4	VTASDPLDTLGSEGALSPGGVASLLR	–0.061	18.006	–1.481	0.140	0.179
CO5	P01031	DINYVNPVIK	–0.092	16.181	–1.264	0.207	0.251
CO5	P01031	TLLPVSKPEIR	–0.070	17.208	–1.043	0.298	0.345
CO5	P01031	VFQFLEK	–0.065	18.209	–0.965	0.335	0.378
CO6	P13671	ALNHLPLEYNSALYSR	–0.109	16.292	–1.689	0.092	0.132
CO6	P13671	SEYGAALAWEK	–0.101	15.755	–1.744	0.082	0.119
CO8B	P07358	IPGIFELGISSQSDR	–0.036	14.471	–0.478	0.633	0.660
CO8B	P07358	SDLEVAHYK	–0.049	13.083	–0.781	0.435	0.472
CO8B	P07358	YEFILK	–0.038	18.876	–0.613	0.541	0.570

associations between *APOE4* copy number and CSF peptide expression levels, correcting for CSF Aβ levels as well as age and gender. In this model, 176 of 294 peptides had statistically significant *APOE4* copy number-related changes in CSF expression levels (Supplementary File 2). Increasing *APOE4*-copy number was associated with increased expression of the *APOE4*-specific peptide (LGADMEDVR) ($q = 1.89 \times 10^{-62}$) and decreased expression of the CRP-derived peptide (ESDTSYVSLK) ($q < 0.045$) after controlling for CSF Aβ levels. Increasing *APOE4* copy number was associated with increased expression of a pan-APOE peptide (LGPLVEQGR) in this model ($q = 1.69 \times 10^{-7}$). As in models 1 and

Table 7
Expression of the Top 20 Indicated Peptides and for all complement peptides/proteins by *APOE4* copy number in multivariate models accounting for age, gender and CSF Aβ levels (Model 5). For full data set, see Supplementary File 2

Top 20 Proteins/Peptides whose Expression differed as function of APOE4 copy number, controlling for age, gender, and Aβ

Protein	UniProt ID	Peptide	Log Fold Change	Average Expression	*t* statistic	p	FDR Corrected p
APOE	P02649	LGADMEDVR	3.664	9.224	23.026	0.000	0.000
APOE	P02649	LGPLVEQGR	0.565	22.385	6.309	0.000	0.000
APOE	P02649	LAVYQAGAR	0.572	25.381	6.007	0.000	0.000
APOE	P02649	AATVGSLAGQPLQER	0.515	20.108	5.631	0.000	0.000
NRX1A	Q9ULB1	ITTQITAGAR	0.240	16.206	4.981	0.000	0.000
AATM	P00505	FVTVQTISGTGALR	0.209	10.293	4.719	0.000	0.000
NRX1A	Q9ULB1	DLFIDGQSK	0.209	16.186	4.688	0.000	0.000
PCSK1	P29120	GEAAGAVQELAR	0.245	21.388	4.676	0.000	0.000
L1CAM	P32004	LVLSDLHLLTQSQVR	0.229	10.874	4.607	0.000	0.000
CADM3	Q8N126	GNPVPQQYLWEK	0.179	15.815	4.557	0.000	0.000
AATC	P17174	NLDYVATSIHEAVTK	0.193	11.536	4.312	0.000	0.000
ALDOA	P04075	ALQASALK	0.202	19.150	4.415	0.000	0.000
ALDOA	P04075	QLLLTADDR	0.190	16.230	4.332	0.000	0.000
CADM3	Q8N126	EGSVPPLK	0.158	16.142	4.328	0.000	0.000
CADM3	Q8N126	SLVTVLGIPQKPIITGYK	0.191	16.167	4.424	0.000	0.000
KPYM	P14618	LDIDSPPITAR	0.214	18.500	4.308	0.000	0.000
NRX1A	Q9ULB1	SDLYIGGVAK	0.189	16.548	4.329	0.000	0.000
PCSK1	P29120	ALAHLLEAER	0.242	19.035	4.329	0.000	0.000
PCSK1	P29120	NSDPALGLDDDPDAPAAQLAR	0.227	13.959	4.351	0.000	0.000
PRDX1	Q06830	DISLSDYK	0.185	14.594	4.377	0.000	0.000

CSF Complement Protein/Peptide Expression as a function of APOE4 copy number, controlling for age, gender, and Aβ

Protein	UniProt ID	Peptide	Log Fold Change	Average Expression	*t* statistic	p	FDR Corrected p
C1QB	P02746	LEQGENVFLQATDK	0.039	17.123	0.983	0.326	0.393
C1QB	P02746	VPGLYYFTYHASSR	0.096	17.588	1.782	0.076	0.114
CFAB	P00751	DAQYAPGYDK	–0.009	16.383	–0.132	0.895	0.904
CFAB	P00751	VSEADSSNADWVTK	–0.051	16.209	–0.956	0.340	0.408
CFAB	P00751	YGLVTYATYPK	0.007	22.512	0.119	0.905	0.911
CO2	P06681	DFHINLFR	0.085	18.670	1.381	0.168	0.232
CO2	P06681	HAIILLTDGK	0.050	15.673	1.008	0.315	0.384
CO2	P06681	SSGQWQTPGATR	0.054	15.901	1.026	0.306	0.378
CO3	P01024	IHWESASLLR	–0.032	13.985	–0.215	0.830	0.853
CO3	P01024	TELRPGETLNVNFLLR	–0.011	10.138	–0.227	0.821	0.847
CO4A	P0C0L4	DHAVDLIQK	0.100	22.139	1.690	0.092	0.135
CO4A	P0C0L4	GSFEFPVGDAVSK	0.140	25.290	2.132	0.034	0.056
CO4A	P0C0L4	LGQYASPTAK	0.122	21.693	1.975	0.049	0.079
CO4A	P0C0L4	NVNFQK	0.100	18.384	1.776	0.077	0.115
CO4A	P0C0L4	VLSLAQEQVGGSPEK	0.055	19.994	1.009	0.314	0.384
CO4A	P0C0L4	VTASDPLDTLGSEGALSPGGVASLLR	0.066	18.007	1.380	0.169	0.232
CO5	P01031	DINYVNPVIK	0.241	16.153	2.987	0.003	0.007
CO5	P01031	TLLPVSKPEIR	0.207	17.184	2.768	0.006	0.012
CO5	P01031	VFQFLEK	0.216	18.187	2.874	0.004	0.010
CO6	P13671	ALNHLPLEYNSALYSR	0.054	16.276	0.731	0.465	0.526
CO6	P13671	SEYGAALAWEK	0.029	15.745	0.434	0.665	0.706
CO8B	P07358	IPGIFELGISSQSDR	0.107	14.458	1.208	0.228	0.298
CO8B	P07358	SDLEVAHYK	0.049	13.073	0.679	0.498	0.560
CO8B	P07358	YEFILK	0.061	18.865	0.844	0.399	0.468

2, all five CH3L1-, FABPH-, and ALDOA-derived peptides also showed statistically significant *APOE4* copy number related increases in expression after correcting for CSF Aβ levels ($q < 0.05$ for each; Table 7).

In this model controlling for CSF Aβ levels (as well as age and sex), all three peptides from complement factor 5 (CO5) showed a statistically significant increase in expression as a function of increasing *APOE4* copy number ($q < 0.05$ for each; Table 7). In contrast to the results from models 1–4, in this model (controlling for CSF Aβ levels) no consistent direction of association was seen between *APOE4* copy number and the other 21 complement-derived peptides (Table 7).

Table 8
Summary of Change Direction for Top proteins and Complement Pathway Proteins in Statistical Models 1 – 5. Up arrow indicates expression increase for the indicated protein as a function of increasing *APOE4* copy number in the given statistical model; down arrow indicates expression decrease for the indicated protein as a function of increasing *APOE4* copy number in the given statistical model

Protein	UniProt ID	Peptide	Key functions	Statistical Model#				
				1	2	3	4	5
CRP	P02741	ESDTSYVSLK	Acute phase reactant [40]. Mediator of inflammatory and apoptotic processes, including activation of classical complement pathway [41], opsonization of atherosclerotic plaques [42].	↓[2]	↓[2]	↓[3]	↓[3]	↓[1]
CH3L1	P36222	ILGQQVPYATK	Glycoprotein thought to modulate tissue remodeling, and angiogenesis, highly expressed in reactive astrocytes after acute and chronic neuroinflammation [63–65]. Also expressed by activated microglia.	↑	↑	↑	↑	↑
		VTIDSSYDIAK						
FABPH	P05413	SIVTLDGGK	Lipid transporter regulates membrane composition and stability [71], may alter the activity of gamma-secretase or slow trans-membrane lipid transport to increase Aβ deposition in *APOE4* carriers [72].			↑		
				↑	↑		↓	↑
		SLGVGFATR				↓		
ALDOA	P04075	ALQASALK	Glycolytic enzyme that breaks down fructose 1-6-diphosphate; decreased glucose metabolism has been observed in the brains of patients with AD [73], likely due to oxidative stress that impairs ATP production, induces synaptic dysfunction, and causes neuronal death [74].	↑	↑	↓	↓	↑[3]
		QLLLTADDR						
Complement pathway proteins								
C1QB	P02746	LEQGENVFLQATDK	Complement pathway activation and complement factor-dependent synaptic phagocytosis thought to represent a neurodegeneration mechanism in AD [82, 83]	↓	↓	↓	↓	↑
		VPGLYYFTYHASSR		↓	↓	↓[2]	↓[1]	↑
CFAB	P00751	DAQYAPGYDK		↓	↓	↓	↓	↓
		VSEADSSNADWVTK		↓	↓	↓	↓	↓
		YGLVTYATYPK		↓	↓	↓[1]	↓[1]	↑
CO2	P06681	DFHINLFR		↓	↓	↓	↓	↑
		HAIILLTDGK		↓	↓	↓[1]	↓[1]	↑
		SSGQWQTPGATR		↓	↓	↓[1]	↓[1]	↑
CO3	P01024	IHWESASLLR		↓	↓	↓	↓	↓
		TELRPGETLNVNFLLR		↓	↓	↓	↓	↓
CO4A	P0C0L4	DHAVDLIQK		↓	↓	↓	↓	↑
		GSFEFPVGDAVSK		↓	↓	↓	↓	↑
		LGQYASPTAK		↓	↓	↓	↓	↑
		NVNFQK		↓	↓	↓	↓	↑
		VLSLAQEQVGGSPEK		↓	↓	↓	↓	↑
		VTASDPLDTLGSEGAL SPGGVASLLR		↓	↓	↓	↓	↑
CO5	P01031	DINYVNPVIK		↓	↓	↓	↓	↑[2]
		TLLPVSKPEIR		↓	↓	↓	↓	↑[1]
		VFQFLEK		↓	↓	↓	↓	↑[2]
CO6	P13671	ALNHLPLEYNSALYSR		↓	↓	↓	↓	↑
		SEYGAALAWEK		↓	↓	↓	↓	↑
CO8B	P07358	IPGIFELGISSQSDR		↓	↓	↓	↓	↑
		SDLEVAHYK		↓	↓	↓	↓	↑
		YEFILK		↓	↓	↓	↓	↑

#Statistical Models: Model 1: *APOE4*-dependent CSF peptide changes and age and gender. Model 2: *APOE4*-dependent CSF peptide changes and age, gender, and AD clinical status. Model 3: *APOE4*-dependent CSF peptide changes and age, gender, and CSF tau levels. Model 4: *APOE4*-dependent CSF peptide changes and age, gender, and CSF p-tau levels. Model 5: *APOE4*-dependent CSF peptide changes and age, gender, and CSF Aβ levels. [1]FDR corrected $p < 0.05$. [2]FDR corrected $p < 0.01$. [3]FDR corrected $p < 0.001$.

DISCUSSION

Here we found that increasing *APOE4* copy number was associated with decreased CSF CRP levels, which remained significant even after controlling for AD clinical status (Model 2), CSF tau levels (Model 3), CSF p-tau levels (Model 4), and/or CSF Aβ levels (Model 5; see Table 8 for summary of changes

across models). In several models, we also found significant *APOE4* copy number-related increases in pan-APOE peptides, and *APOE4* copy number-related increases in peptides from CH3L1, FABPH, and ALDOA. Finally, except when controlling for CSF Aβ levels in model 5, in all other models a consistent inverse direction of association was observed between increasing *APOE4* copy number and CSF levels of all 8 complement proteins measured int his targeted proteomics dataset.

One of the most consistent findings across all five statistical models examined here was that increasing *APOE4* copy number was associated with reduced CSF levels of the CRP-derived peptide (ESDTSYVSLK, see Table 8). CRP is an acute phase reactant [40] and acts as a mediator of inflammatory and apoptotic processes, including activation of the classical complement pathway [41] and opsonization of atherosclerotic plaques [42]. Recent prospective studies have found that elevated serum CRP levels in midlife may predict increased AD risk, though there is paradoxical shift in which CRP levels decline with advancing age and before clinical AD symptoms appear [43–45]. Further, reduced CRP levels in peripheral blood [46–49] and in CSF [50, 51] correlate with increased cognitive dysfunction and further AD progression in an *APOE4*-dependent manner. These findings fit well with our observation of a significant decrease in CSF CRP levels as a function of increasing *APOE4* copy number and suggests that reduced CRP levels may play a key biological role in *APOE4*-induced increased AD risk.

CRP is typically viewed as a marker of inflammation, so a straightforward interpretation of these results would be that increasing *APOE4* copy number is associated with decreased CNS inflammation, which could play a role in increased AD risk if *APOE4* leads to a reduction in inflammatory processes involved in clearing amyloid plaques or other neurotoxic pathology. Alternatively, although elevated CRP levels are typically viewed as indicative of active inflammation, low and low-normal CRP levels have been found in chronic inflammatory conditions with active disease such as lupus [52], rheumatoid arthritis [53], and inflammatory bowel disease [54, 55]. Thus, the reduced CSF CRP levels observed here may similarly reflect chronically increased inflammation within the CNS of *APOE4* carriers, which could play a role in neurodegeneration and AD risk. CRP has been also implicated in the early development of amyloid plaque formation, neuronal damage, and AD risk [53–56]. The decreased CSF CRP levels observed here may reflect CRP deposition in Aβ plaques and neurofibrillary tangles, in which CRP might play a role in promoting the development of AD. Low CSF CRP levels could also reflect increased CRP consumption from opsonin-mediated glial phagocytosis in AD pathology, as previously suggested [62]. In this way, CSF CRP reductions might alternatively reflect a compensatory glial-dependent Aβ removal process in *APOE4* carriers. Future studies should thus determine whether lower CSF CRP levels in *APOE4* carriers represent a mechanism of neurodegeneration or a compensatory process against it, or even an *APOE4*-copy related process unrelated to the development of AD.

All five models studied here also demonstrated a strong positive correlation between *APOE4* copy number and CSF expression of the *APOE4* allele specific peptide LGADMEDVR [37, 38], which serves as a strong internal control for the validity of this dataset. We also found that increasing *APOE4* copy number was associated with increased CSF pan-APOE peptide levels when controlling for AD clinical status or CSF Aβ levels (Models 2 and 5, respectively) but not when controlling for either CSF tau or CSF p-tau levels (Models 3 and 4, respectively). Taken together, these results are consistent with a paradigm in which *APOE4* allele leads to increased CSF ApoE protein levels before and during the phase in which patients are developing brain Aβ pathology (as measured here by CSF Aβ levels). Then, as frank neurodegeneration begins (as evidenced by rising CSF tau and p-tau levels), there is no longer a significant increase in CSF ApoE protein levels as a function of increasing *APOE4* allele count. This could help explain conflicting prior studies on CSF ApoE protein levels in *APOE4* carriers [57–59], since it suggests that the relationship between *APOE4* copy number and CSF ApoE protein levels would change as neurodegeneration begins (i.e., as defined by increasing CSF tau and p-tau levels). Yet, it is unclear whether the increased AD risk in APOE4 carriers is due to *APOE4* copy number-related increases in ApoE protein levels versus functional or structural changes in the *APOE4* allele-encoded ApoE protein. Indeed, it remains debated in the field to what extent *APOE4*–related increased AD risk represents a toxic gain of function(s) or a loss of protective function(s) (reviewed in [60]).

In models 1 and 5, but not in models 2–4 (Table 8), increasing *APOE4* copy number was also associated with increased expression of peptides derived from CH3L1 (also known as YKL-40) ($q < 0.05$),

FABPH ($q < 0.05$), and ALDOA ($q < 0.05$), as seen in another recent paper [61]. These findings suggest that *APOE4* copy number is associated with increased CSF CH3L1, FABPH, and CH3L1 levels independent of CSF Aβ level changes, but these increases dissipate after controlling for neurodegeneration (i.e., via rising CSF tau or p-tau levels) or by controlling for neurodegeneration-related clinical status (i.e., MCI or dementia).

Our finding of *APOE4*-related increases in CSF CH3L1 levels is corroborated by another recent study using ELISA assays that found increased CSF CH3L1 levels in *APOE4* carriers [62], CH3L1 is a glycoprotein hypothesized to modulate tissue remodeling, and angiogenesis and is highly expressed in reactive astrocytes after acute and chronic neuroinflammation [63–65]. After traumatic brain injuries, *CH3L1* knockout mice demonstrated greater astrogliosis, immune cell infiltration, and neurologic impairment compared to wild-type mice, suggesting CH3L1 plays a role in regulating neuroinflammation [66]. Astrocytes are known regulators of neuroinflammation and oxidative stress in the nervous system, and CH3L1-expressing astrocytes have also been shown to cluster around Aβ plaques and vessels with β-amyloid angiopathy in AD [67]. Taken together, these findings and the data presented here suggest that CH3L1 may contributes to pathologic astrocyte activation in *APOE4* carriers while Aβ pathology is developing, prior to actual neurodegeneration.

Our finding of a positive correlation between *APOE4* copy number and CSF FABPH levels when controlling for Aβ levels, but not when controlling for CSF neurodegeneration markers (i.e., CSF tau and p-tau; Table 8), is consistent with prior studies suggesting a role for FABPH in AD [29, 37, 68–70]. FABPH is a lipid transporter that regulates membrane composition and stability [71], and may alter the activity of gamma-secretase or slow transmembrane lipid transport to increase Aβ deposition in *APOE4* carriers [72]. Indeed, prior studies have found that CSF FAPBH levels in *APOE4* carriers are associated with low CSF Aβ levels and atrophy of the entorhinal cortex and other AD-vulnerable brain regions [69]. These findings indicate a potential role for FABPH in *APOE4*-mediated Aβ accumulation and later neurodegeneration. The findings presented here further support this idea, and further suggest that altered FABPH levels likely play a role in the development of early Aβ pathology in *APOE4* carriers, before CSF tau and p-tau levels begin to decline.

We also found that increasing *APOE4* copy number was associated with increased CSF ALDOA levels in models one and five (Table 8). ALDOA is a glycolytic enzyme that catalyzes the breakdown of fructose 1-6-diphosphate, which is interesting because decreased glucose metabolism has been observed in the brains of patients with AD [73], likely due to oxidative stress that impairs ATP production, induces synaptic dysfunction, and causes neuronal death [74]. A recent study has also proposed that CSF ALDOA levels are a sensitive and specific biomarker of cognitive impairment due to AD [75]. Thus, our finding of an *APOE4* copy number-dependent increase in CSF ALDOA levels may reflect altered glucose metabolism within the CNS of *APOE4* carriers, which has also been observed in AD. It is unclear whether increased ALDOA levels are part of the mechanism for impaired glucose metabolism in *APOE4* carriers and in AD, or whether these increased ALDOA levels reflects a secondary biologic compensation as the result of dysregulated glucose metabolism in *APOE4* carriers. As in the case of CH3L1 and ALDOA, the data presented here suggest that *APOE4* copy number is likely associated with increased CSF ALDOA levels while individuals are clinically normal, and either completely free of AD neuropathology or when they show CSF evidence of Aβ, but not tau or p-tau, pathology.

Further, while not statistically significant, there was a consistent inverse direction of association between *APOE4* copy number and expression of all 24 peptides from all 8 different complement proteins measured (Fig 1A, B) in model 1. These findings were present even after controlling for AD clinical status in model 2, suggesting that the lower complement levels seen as a function of *APOE4* copy number do not simply reflect confounding due increased AD dementia frequency in *APOE4* carriers. Further, this consistent inverse direction of association between *APOE4* copy number and expression of all 24 peptides from all 8 different complement proteins measured here was also observed after controlling for CSF tau and p-tau levels in models 3 and 4, respectively. These findings suggest that that these potential *APOE4*-related reductions in CSF complement levels are not due to confounding from neurodegeneration in *APOE4* carriers.

Nonetheless, this consistent inverse direction of association between *APOE4* copy number and CSF complement protein expression was not observed in model 5, which controlled for CSF Aβ levels. These findings would fit with a scenario in which

APOE4 leads to reductions in both CSF Aβ levels and CSF complement protein levels, and these *APOE4*-dependent reductions in CSF Aβ and complement levels are closely associated with one another (or co-linear). In this case, increasing *APOE4* copy number would be expected to be associated with reduced complement levels in all situations except when controlling for falling CSF Aβ levels. This framework is consistent with the finding that complement proteins deposit into Aβ plaques [76, 77], which would offer a potential biochemical explanation for a co-linear relationship between reductions in CSF Aβ and complement levels as a function of increasing *APOE4* copy number.

The finding that *APOE4* copy number may be associated with decreased CSF complement protein levels is supported by two other recent studies that also found lower CSF complement protein levels in *APOE4* carriers [61, 78]. While the absolute magnitude of the reduction in each complement-protein derived peptide was modest, the chance that the level of 24 peptides (or 8 proteins) would all decrease due to chance alone is extremely low, suggesting that this likely represents a true biological finding. These lower complement protein levels could represent either decreased complement factor transcription/translation or increased degradation in *APOE4* carriers. The former possibility is unlikely, though, because prior work has shown that *APOE4* is not associated with alterations in the transcription or translation of complement pathway proteins [79]. These data therefore suggest that increasing *APOE4* copy number may be associated with increased complement pathway protein degradation in the CNS.

Complement protein degradation can be caused by complement cascade activation, which involves cleavage and degradation of complement proteins [80]. Thus, the relationship between increasing *APOE4*-copy number and potential reductions in CSF complement protein levels may reflect increased complement pathway activation. Recent work suggests that the ApoE protein is a negative regulator of complement pathway activation [81]. Taken together with our results, this raises the possibility that the *APOE4* allele results in reduced inhibition (i.e., disinhibition) of the complement pathway, thus resulting in *APOE4* copy number-dependent reductions in CSF levels of complement pathway proteins (due to complement degradation).

Complement pathway activation and complement factor-dependent synaptic phagocytosis is thought to represent a neurodegeneration mechanism in AD [78, 82]; thus, our results raise the possibility that *APOE4* may contribute to AD risk by increasing complement pathway activation and resultant synaptic phagocytosis and neurodegeneration. Overall, even though the *APOE4*-related reductions in CSF complement protein levels seen here were generally not statistically significant after multiple comparison correction, the convergence of our human findings with data from cellular [79] and mouse models [83] suggest that further studies are warranted on the relationship between *APOE4* copy number and CSF complement protein levels.

This work has several limitations. First, the data set studied here included very few African-American *APOE4* allele carriers, raising the question of how well these data may generalize to African-American or other non-Caucasian populations. Future work should study the effects of the *APOE4* allele on the CSF proteome in other racial and ethnic populations to develop a broader understanding of how *APOE4* may increase AD risk. Second, the data analyzed here were originally obtained to study CSF proteomic correlates of dementia due to AD or MCI [29], rather than to study the effects of *APOE4* copy number on the CSF proteome. Although we controlled for clinical status in model 2, the relatively smaller number of study patients within each clinical status cohort (i.e., normal, MCI, or dementia due to AD) likely limited statistical power to detect effects of *APOE4* on the CSF proteome itself. Thus, future studies on this topic should focus on larger clinically homogenous study populations (i.e., all cognitively normal individuals, or all individuals with dementia due to AD) to reduce variance within each genotype group, and to improve statistical power. Third, the data reported here were from a targeted proteomic platform that only measured 222 proteins total, including just 8 of the over 30 proteins in the full complement pathway [84]. Future studies should focus on quantitating the CSF levels of a broader array of proteins including all complement pathway protein to develop a more complete understanding of the relationship between *APOE4* allele copy number and the classical, lectin and alternative complement cascades and other protein pathways.

Nonetheless, the data presented here provide strong support for the hypothesis that the increased AD risk in *APOE4* carriers is related to changes within CRP-related biological processes, and to changes in biological mechanisms involving CH3L1, FABPH, ALDOA, and the complement pathway. The results from our five different statistical models also

demonstrate key differences across the neuropathologic phases of preclinical AD in the CSF proteomic correlates of *APOE4* copy number. Thus, future CSF proteomic studies designed to identify the earliest mechanisms by which *APOE4* contributes to AD risk may need to focus on patients without significant Aβ or neurodegenerative pathology.

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SUPPLEMENTARY MATERIAL

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