Bi-directional Association of Cerebrospinal Fluid Immune Markers with Stage of Alzheimer's Disease Pathogenesis

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Abstract. Immune mechanisms may be important in the pathogenesis of Alzheimer's disease (AD). Yet, studies comparing cerebrospinal fluid (CSF) and plasma immune marker levels of healthy and demented individuals have yielded conflicting results. We analyzed CSF from 101 members of the parental history-positive PREVENT-AD cohort of healthy aging adults, and 237 participants without dementia from the initial cohort of the Alzheimer's Disease Neuroimaging Initiative (ADNI-1). Following recent practice, we used the biomarkers total-*tau* and amyloid-β_{1-42} to allocate participants from each study into four stages of AD pathogenesis: Stage 0 (no abnormality), Stage 1 (reduced amyloid-β_{1-42}), Stage 2 (reduced amyloid-β_{1-42} and increased total-tau), or "Suspected Non-Alzheimer Pathology" (elevated total-tau only). Investigating the PREVENT-AD participants' CSF assay results for 19 immune/inflammatory markers, we found six that showed a distinct bi-directional relationship with pathogenetic stage. Relative to Stage 0, these were diminished at Stage 1 but strongly increased at Stage 2. Among the ADNI participants (90 healthy controls and 147 with mild cognitive impairment), we found that 23 of 83 available CSF markers also showed this distinct pattern. These results support recent observations that immune activation may become apparent only after the onset of both amyloid and tau pathologies. Unexpectedly, they also suggest that immune marker activity may diminish along with earliest appearance of amyloid-β plaque pathology. These findings may explain discordant results from past studies, and suggest the importance of characterizing the extent of AD pathology when comparing clinical groups.

Keywords: Alzheimer's disease, biomarkers, inflammation, pathology

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by extracellular amyloid-β (Aβ) plaques, intracellular *tau* neurofibrillary tangles, and widespread synapse loss. A decades-long period of pathogenetic change precedes the appearance of dementia [1–3]. Immune mechanisms are thought to be important in this process [4]. In the early 1990s, McGeer et al. [5] observed that co-existence of AD and rheumatoid arthritis (RA) was rare in autopsy data. These investigators had earlier observed that inflammatory processes were vigorous in the vicinity of AD lesions [6]. Because RA is often treated with anti-inflammatory drugs, they suggested that the latter might explain the low prevalence of AD changes in RA patients. Ensuing epidemiological studies identified an inverse relationship between the use of non-steroidal anti-inflammatory drugs and incidence of AD (for review, see [4]). More recently, genetic studies have identified immune/inflammatory pathways as modifiers of AD dementia risk [7–10]. Inflammatory changes are therefore thought to be a response to appearance of AD pathology, increasing linearly with disease progression. It is widely assumed that such inflammatory activity is harmful.

In fact, this last notion has not been verified *in vivo* by measures in plasma or cerebrospinal fluid (CSF) [11]. Instead, investigations in fully penetrant autosomal dominant familial AD suggest that significant increases in immune signaling may occur only after the appearance of both Aβ and *tau* pathologies [12]. Similarly, other studies of the association of single CSF immune markers and AD pathology have suggested that the former decline concurrently with reduction in $A\beta_{1-42}$ concentration that typifies early AD pathogenesis [13–15]. Here we describe multi-analyte investigations of the association between AD pathogenesis, as revealed by CSF biomarkers, and several immune/inflammatory proteins. We report initial observations in the high-risk PREVENT-AD cohort of healthy older persons, followed by analyses of CSF from the Alzheimer's Disease Neuroimaging Initiative (ADNI-1). Our objective was to determine whether variations in AD pathological "stage" [16, 17] related to CSF immune and other protein levels, and whether such varied association could explain conflicting results from past studies.

METHODS

Participants

PREVENT-AD participants (Table 1) were 101 cognitively intact volunteers with a parental or multiple-sibling history of "sporadic" AD [18]. They scored an education-adjusted ≥23/30 on the Montreal Cognitive Assessment [19], and had little if any difficulty with subsequent cognitive testing. Most were 60 years of age or older, but persons aged 55–59 years were eligible if their age was within 15 years of their youngest-affected relative's onset. Each participant and study partner provided written informed consent. All procedures were approved by the McGill University Faculty of Medicine Institutional Review Board. All research complied with ethical principles of the Declaration of Helsinki.

ADNI-1 data were downloaded in February 2016 from http://adni.loni.usc.edu. ADNI was launched in 2003 as a public-private partnership led by Principal Investigator Michael W. Weiner. Its primary goal has been to test whether serial magnetic resonance imaging, positron emission tomography (PET), and various clinical, biological and neuropsychological markers can be combined to measure progression of MCI and early AD. Given our interest in analyses of ADNI participants before onset of dementia, we limited analyses to 237 ADNI-1 participants with available CSF data from the healthy control (*n* = 90) and MCI groups (*n* = 147), the latter to enrich for persons with likely Aβ or *tau* pathology (Table 1).

Cerebrospinal fluid measurements

Lumbar punctures (LPs) in PREVENT-AD volunteers were performed following an overnight fast using the Sprotte 24-gauge atraumatic needle. Samples of 20–30 mL were aliquoted (500 μL) into propylene cryotubes and stored at –80°C. We used procedures from the BIOMARK-APD consortium of the EU Joint Program in Neurodegenerative Diseases, to measure CSF concentrations of the AD biomarkers $A\beta_{1-42}$, total-*tau* (t-*tau*) and P_{181}-*tau* (P-*tau*) using the Innotest enzyme-linked immunosorbent assay kit (Fujirebio, Ghent, Belgium). CSF apolipoprotein E (apoE) levels were assayed using the Milliplex APOMAG-62k human apolipoprotein cardiovascular disease multiplex kit (EMD-Millipore, Billerica, MA, USA).

Table 1
PREVENT-AD and ADNI demographics

	PREVENT-AD					ADNI-1				
	All participants	Stage 0	Stage 1	Stage 2	SNAP	All participants	Stage 0	Stage 1	Stage 2	SNAP
Sample	101[‡]	51	12	9	10	237	78	71	79	9
Age Mean (sd)	62.90 (5.56)	62.80 (5.31)	60.91 (5.52)	63.99 (4.39)	64.52 (8.66)	75.26 (6.68)	75.24 (6.71)	75.48 (6.09)	74.77 (7.40)	77.89 (4.04)
% Male	30.69	35.29	25	55.56	30	61.60	64.10	61.97	62.03	33.33
% *APOE4*-positive	36.63	31.37	50	88.89	20	42.19	10.26	52.11	68.35	11.11
Clinical Diagnostic Category (HC: MCI)	101: 0	51: 0	12: 0	9: 0	10: 0	90: 147	49: 29	23: 48	11: 68	7: 2
Education (y) Mean (sd)	14.88 (2.93)	15.00 (2.89)	14.92 (2.35)	13.67 (3.32)	15.1 (1.59)	15.83 (2.94)	15.82 (2.76)	15.76 (3.15)	15.73 (2.94)	17.33 (2.79)
CSF Aβ_{1-42} (pg/mL) Mean (sd)**	1062.91 (280.65)	1161.58 (158.54)	705.02 (115.67)	611.35 (85.68)	1422.15 (113.63)	178.50 (56.06)	244.41 (24.71)	146.44 (22.31)	133.95 (21.90)	251.20 (28.32)
CSF t-tau (pg/mL) Mean (sd)**	273.09 (129.97)	221.94 (56.14)	177.59 (73.16)	522.45 (162.64)	435.48 (90.79)	92.17 (49.85)	58.08 (15.30)	67.47 (17.38)	146.15 (47.97)	108.58 (12.95)
CSF P-tau (pg/mL) Mean (sd)**	46.83 (18.00)	40.25 (10.37)	32.70 (10.71)	75.47 (19.89)	72.29 (11.45)	31.91 (15.94)	20.14 (7.20)	27.20 (13.00)	47.83 (11.95)	31.33 (8.92)

pg/mL, picograms per milliliter; sd, standard deviation; %APOE, proportion of *APOE* ε4 carriers; SNAP, Suspected Non-Alzheimer Pathology; HC, healthy controls; MCI, mild cognitive impairment; **ADNI and PREVENT-AD used different assays to measure AD biomarkers. [‡] To enhance contrast, 19 individuals within $\pm$5% of inter-stage thresholds were not assigned to a stage, except as noted in text. See text also for definition of the several stages.

To characterize immune/inflammatory status, we attempted to assay CSF concentrations of 45 cytokines and chemokines using a combination of the Milliplex HCYTMAG60PMX29BK xMap kit (EMD-Millipore; Supplementary Methods) and the Mesoscale V-plex neuro-inflammation panel-1 (Mesoscale Discovery, Rockville, MD). Detailed procedures for the latter have been described elsewhere [20]. We excluded measures that fell outside the useful assay range. We also excluded markers with excessive assay variation, as measured by a mean coefficient of variation exceeding 15%. We then performed an outlier analysis to identify and remove from consideration assay values above the third or below the first quartile $\pm$ 1.5 times the interquartile distance [21]. After applying these QC restrictions, there remained 10 markers (out of 21 initially) that were assayed using both Mesoscale and Luminex technologies. We compared the two techniques' agreement and range of values for each analyte. When necessary, we chose the marker assay with more quality-control acceptable readings or without ceiling or floor effects. We excluded analytes having ≤ 50 quality-control-acceptable readings from at least one assay technique. Nineteen immune marker assays met these standards.

The ADNI investigators had measured CSF $A\beta_{1-42}$ and t-*tau* concentrations using Research Use Only INNOBIA AlzBio3 immunoassay reagents (Fujirebio) on an xMap Luminex platform (http://adni.loni. usc.edu/methods/biomarker-analysis/). CSF levels of 83 other proteins had been assayed via a multiplex x-Map kit from Rules Based Medicine (MyriadRBM, Austin, TX). Rigorous quality control standards excluded markers with >10% missing data. Results were then normalized using boxcox transformation, etc. (for details, see http://adni.loni.usc.edu/wp-cont ent/uploads/2012/01/2011Dec28-Biomarkers-Consor tium-Data-Primer-FINAL1.pdf.)

Pathological staging of participants

Following recent convention [16, 17], we used CSF biomarker data to characterize extent of AD pathology in both PREVENT-AD and ADNI. Disease progress was classified as Stage 0 (normal CSF $A\beta_{1-42}$ and t-*tau*), Stage 1 (low $A\beta_{1-42}$, low t-*tau*), Stage 2 (low $A\beta_{1-42}$ and elevated t-*tau*), and Suspected Non-Alzheimer Pathology (SNAP, high t-*tau* only). Very few PREVENT-AD participants met the typical "below-normal amyloid" threshold

of 550 pg/mL. However, that threshold is intended to discriminate healthy controls from subjects with AD dementia, and may be too stringent for identification of persons with early $A\beta$ pathology [22]. Given recent evidence that increasing amyloid burden (reflected by decreasing CSF amyloid concentration) portends poorer clinical outcomes even before typical thresholds are crossed [23–25], we chose a more sensitive cut-off value for $A\beta_{1-42}$ abnormality at the 25th percentile value or <870 pg/mL. Similarly, we specified a t-*tau* abnormality threshold at the 75th percentile, or >335 pg/mL (Fig. 1A). Since ADNI analyses, which used different assay methods, had included individuals with MCI we adopted the recommended $A\beta$ and tau thresholds of <192 pg/mL and >93 pg/ml (Fig. 1B) [26]. Because our PREVENT-AD thresholds were somewhat arbitrary, and because we wished if possible to increase contrasts between stages, we decided *a priori* to exclude data from 19 PREVENT-AD individuals whose marker concentrations were within $\pm 5\%$ of either the $A\beta$ or tau threshold.

APOE genotyping

In PREVENT-AD, *APOE* genotype was determined using the PyroMark Q96 pyrosequencer (Qiagen, Toronto, ON, Canada). DNA was amplified using RT-PCR with primers rs429358 amplification forward 5'-ACGGCTGTCCAAGGAGCT G-3', rs429358 amplification reverse biotinylated 5'-CACCTC GCCGCGGTACTG-3', rs429358 sequencing 5'-CGGACATGGAGGACG-3', rs7412 amplification forward 5'-CTCCGCGATGCCGATGAC-3', rs7412 amplification reverse biotinylated 5'-CCCCGGCC TGGTACACTG-3' and rs7412 sequencing 5'-CGA TGACCTGCAGAAG-3'. In ADNI, *APOE* genotypes had been determined using DNA extracted by Cogenics (Beckman-Coulter, Pasadena, CA) [27].

Statistical analyses

In PREVENT-AD, we used multiple linear regression to examine the association of standardized (z-score) concentrations of the 19 immune markers or apoE with pathological stage. To examine agreement of our results with prior studies, we assessed association of these same marker scores with the individual CSF AD biomarkers $A\beta_{1-42}$, t-*tau*, P_{181}-*tau*, and the t-*tau*/$A\beta_{1-42}$ ratio. To meet model assumptions, we log-transformed P-*tau* and
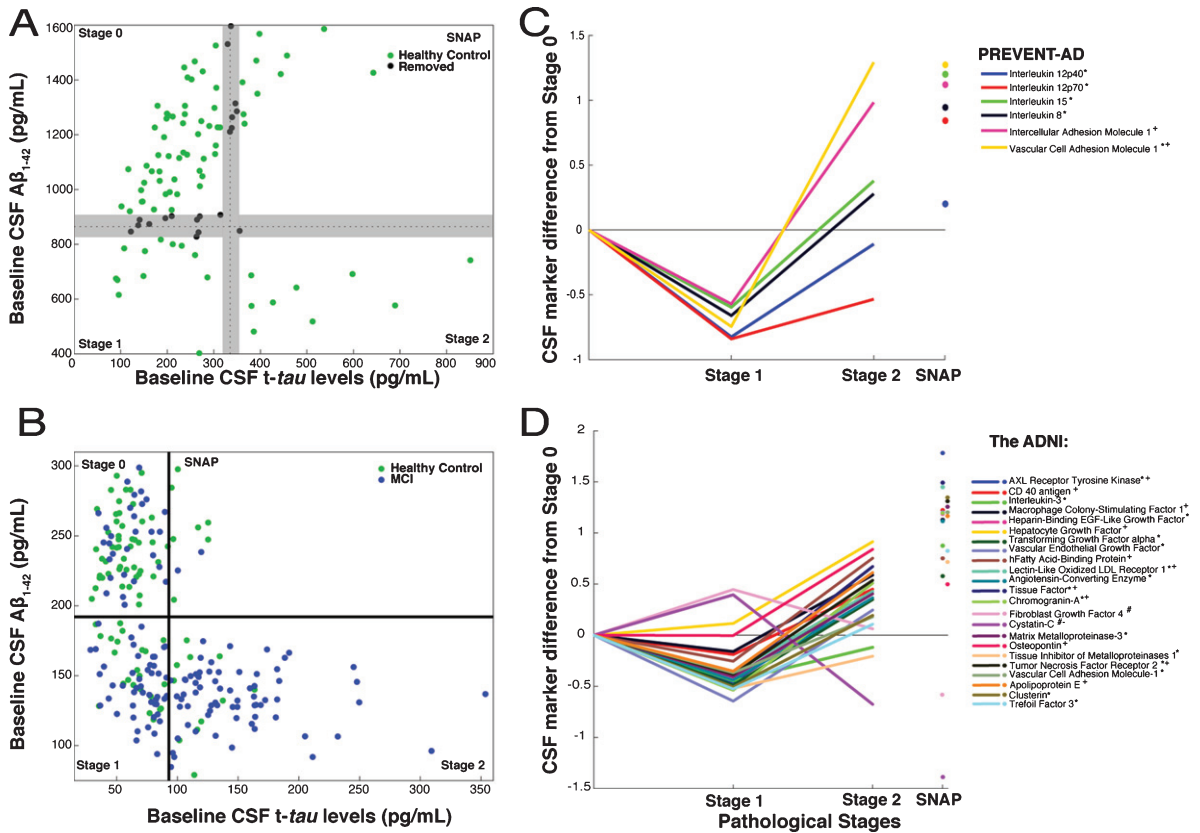
Fig. 1. CSF markers and pathological staging of Alzheimer's disease. A) 101 healthy PREVENT-AD participants were classified as Stage 0, 1, 2, or SNAP based on $A\beta_{1-42}$ (869.75 pg/mL) and t-*tau* (334.6 pg/mL) thresholds. Among them, to enhance contrasts, 19 (black) having CSF $A\beta$ and t-*tau* levels within $\pm 5\%$ of inter-stage thresholds were removed *a priori* from consideration. B) ADNI healthy-control (green) and MCI (blue) participants were similarly classified using the project's recommended thresholds for $A\beta_{1-42}$ (192 pg/mL) and t-*tau* (93 pg/mL). C, D) Linear models, adjusted for participants' age, gender, *APOEε*4 carrier status and (for ADNI only) clinical diagnostic category, were used to assess CSF protein marker level differences by stage. CSF marker data were standardized using z-scores. The β coefficients for stage differences from these models are represented. In PREVENT-AD (C), six CSF markers were associated bi-directionally with pathological stage. Five were lower (*) in Stage 1 versus Stage 0, and two were greater (+) in Stage 2 versus Stage 0 ($p \leq 0.05$ uncorrected). In ADNI (D), 23 CSF markers were either increased (#) or decreased (*) at Stage 1 and at Stage 2 (+ or −) vs. Stage 0. All 23 were significantly different at Stage 2 versus Stage 1 ($p_{FDR} \leq 0.05$).

t-*tau*, and used boxcox transformation to normalize the t-*tau*/Aβ ratio. Models were adjusted for participant age, gender, and *APOE ε*4 carrier status. *p*-values were corrected, as elsewhere, for multiple comparisons using the false discovery rate (FDR) method [28] specifying two-sided $\alpha = 0.05$. Analyses relied on Matlab (Mathworks Inc.; Natick, Massachusetts), SPSS24 (IBM Corp.; Armonk, NY) or R v3.2.2.

We then tested the robustness of our PREVENT-AD findings in the ADNI-1 cohort. To deal with the multiple available ADNI protein markers, we used Bayes factor analysis, employing Matlab's CGBayesNets [29], to identify and remove molecular

species unlikely to be associated with pathological stage. The Bayes' Factor is the ratio of posterior likelihoods of the data being associated with *vs.* independent of pathological stage [30]. We kept only individual markers with a (logarithmic) Bayes' Factor >0, thus providing any evidence in favor of an association between marker levels and pathological stage. Multiple linear regression models then identified markers that differed by stage after adjustment for participant age, gender, *APOE ε*4 carrier status and clinical diagnostic group. As in PREVENT-AD, we also used regression models to assess association of these markers with $A\beta_{1-42}$, t-*tau*, P_{181}-*tau*, and the t-*tau*/$A\beta_{1-42}$ ratio.

RESULTS

Demographic characteristics

Demographic characteristics of both the PREVENT-AD and ADNI-1 participants are summarized in Table 1. Whereas PREVENT-AD included 70% women, ADNI-1 had a majority of men. ADNI participants were also more than a decade older on average. The table indicates the two groups' distributions by pathological stage. As expected, ADNI included larger proportions of persons at Stages 1 or 2.

PREVENT-AD immune marker concentrations and pathological stage

After categorizing PREVENT-AD participants by pathological stage, FDR-adjusted models revealed only trend-level differences in the 20 analyzed markers when comparing Stage 0 versus Stage 1 or Stage 2. Inference here was limited, however, by the small number of individuals at Stages 1 and 2. Omitting FDR adjustment, we observed a consistent bi-directional pattern of association between six marker scores and pathological stage, with reduced concentrations at Stage 1 and/or increased levels at Stage 2 (Table 2, Fig. 1C). Because a *post hoc* power analysis indicated that we had less than the desired 90% power to observe a statistically significant effect for IL-12p40, however, results with this latter marker should be interpreted cautiously. As well, IL-15, IL-8, ICAM-1, and VCAM-1 showed significant elevation at Stage 2 *vs.* Stage 1 (Fig. 1C). Despite the small size of the SNAP sample, this group showed elevation after FDR adjustment in all of the above-mentioned markers as well as granulocyte colony-stimulating factor (GCSF), interferon gamma-induced protein 10 (IP-10), and IL-16 (Supplementary Table 1).

PREVENT-AD immune marker concentrations and AD biomarkers

In corroborative analyses, we found that apoE and seven of the 19 CSF immune markers in PREVENT-AD increased with t-*tau*/Aβ$_{1-42}$ ratio ($p_{FDR} \leq 0.05$, Supplementary Table 2). Levels of these markers increased especially with t-*tau* and P-*tau*. Interestingly, four markers (apoE, IL-15, IL-8, and IL-12p70) also increased with Aβ concentration, suggesting reduced plaque burden [31, 32]. Although unexpected, this association between increasing levels of

immune markers and Aβ has noted before [12–15]. It provides one possible explanation for the bi-directional pattern in Fig. 1C.

ADNI protein marker concentrations, pathological stage, and AD biomarkers

Given the limited size of the PREVENT-AD sample, we wished to verify whether this bi-phasic pattern generalized to other proteins assayed in CSF. Thus, we pursued similar analyses in ADNI-1, including participants with MCI. Bayes factor analysis reduced the 83 ADNI CSF protein markers to 38 with any likelihood of association to pathological stage (Fig. 2). Multiple linear regression modeling then showed that 23 of these differed significantly between Stage 0 and Stage 1 or Stage 2 ($p_{FDR} \leq 0.05$; Fig. 1D, Supplementary Table 3). Marker results by stage again revealed bi-directional patterns, with all but two markers showing reduction at Stage 1 and/or increase at Stage 2. Exceptions were Cystatin-C and fibroblast growth factor-4 (FGF-4), which showed the opposite pattern. Importantly, all remaining markers were increased at Stage 2 *vs.* Stage 1. As in PREVENT-AD, all but three of these (IL-3, tissue inhibitor of metalloproteinase-1, and FGF-4) correlated overall with t-*tau*/Aβ$_{1-42}$ ratio (Supplementary Table 4).

Only three markers (apoE, VCAM-1, and vascular endothelial growth factor [VEGF]) were common to analyses of the PREVENT-AD and ADNI samples. In both groups, VCAM-1 and apoE correlated well with t-*tau*/Aβ$_{1-42}$ ratio (Fig. 3) and showed similar patterns of association with pathological stages, although results were less robust in PREVENT-AD. These associations failed to generalize for VEGF (Supplementary Tables 1 and 3). *APOE* carrier status and clinical diagnostic group did not contribute in our models, as illustrated in Table 3 and Fig. 2.

DISCUSSION

Immune processes are important in the pathogenesis of AD [4]. For example, activated microglia and astrocytes co-localize with amyloid plaques in the brains of AD animal models and individuals with dementia [6].

In the high-risk but cognitively unimpaired PREVENT-AD cohort, we observed a distinct bi-directional relationship between six immune marker levels and pathological stage, an approximate indicator of disease progression. This non-linear pattern appeared despite a direct overall correlation between

Table 2
PREVENT-AD and ADNI cerebrospinal fluid markers associated with Alzheimer's disease pathological stages

| PREVENT-AD	ADNI	
IL-12p40	AXL Receptor Tyrosine Kinase	Chromogranin A
IL-12p70	CD40 antigen	Cystatin-C
IL-8	IL-3	Fibroblast Growth Factor 4
IL-15	Macrophage Colony Stimulating Factor-1	Matrix Metalloproteinase-3
Soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1)	Heparin-Binding EGF-Like Growth Factor	Osteopontin
Soluble Intercellular Cell Adhesion Molecule-1 (sICAM-1)	Hepatocyte Growth Factor	Tissue Inhibitor of Metalloproteinases-1
	Transforming Growth Factor α	Tumor Necrosis Factor Receptor-2
	Vascular Endothelial Growth Factor	Vascular Cell Adhesion Molecule-1
	Heart Fatty Acid Binding Protein	Apolipoprotein-E
	Lectin like Oxidized LDL-Receptor-1	Clusterin (Apolipoprotein J)
	Angiotensin Converting Enzyme	Trefoil Factor-3
	Tissue Factor	
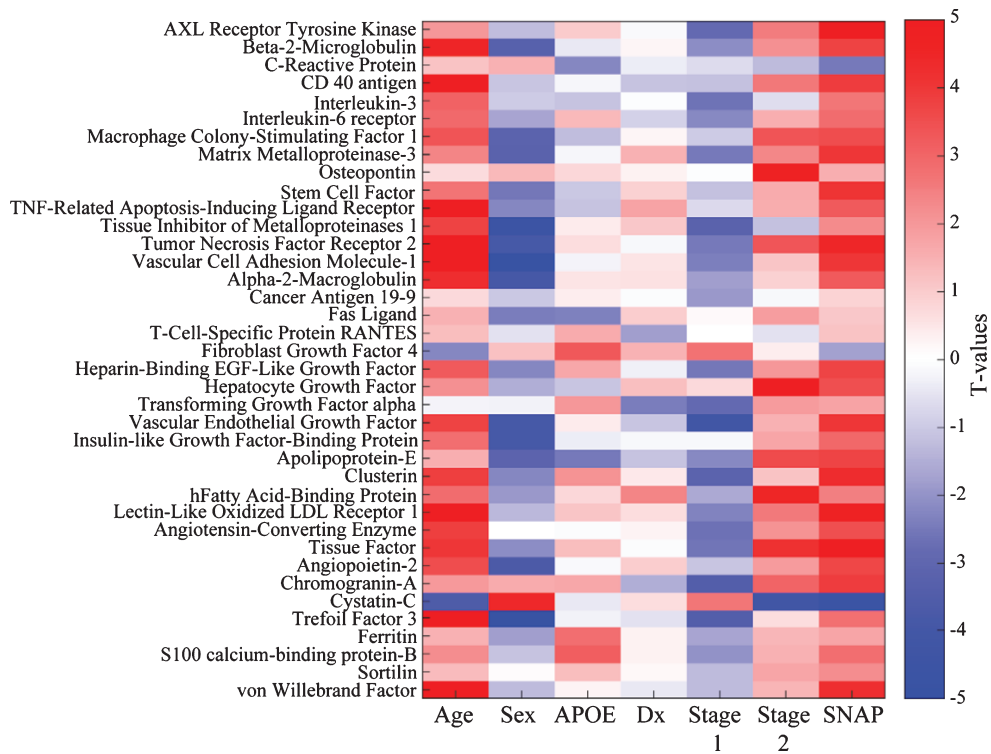
Fig. 2. Bayes factor analysis identifies 38 ADNI CSF markers potentially associated with pathological stage. Shown is a matrix representation of linear regression models predicting association of 38 CSF marker levels with pathological stage with adjustment for age, gender, clinical diagnostic category, and *APOE* status. *T*-values for each association are reported. For categorical variables, the results are shown as comparisons against a reference. For gender, the reference category is male; for *APOE* it is non-carriage of $\varepsilon4$; for diagnosis (Dx), healthy control; and for pathological stage, Stage 0 (no abnormality).

four of these markers with t-*tau*/Aβ_{1-42} ratio. In a corroborative analysis of the ADNI-1 participants (90 HCs and 147 with MCI) having CSF measurements for 83 proteins, we identified a similar pattern of change with advancing AD pathology. As described previously by others [12–15], CSF immune marker levels increased overall with advancing AD

pathology in both cohorts. However, an important refinement of this trend suggested that such marker levels increased strongly only after appearance of *tau* abnormality. Indeed, a number of CSF proteins, including immune markers, appeared to decrease with decline in Aβ_{1-42} (*i.e.*, with increasing plaque burden).
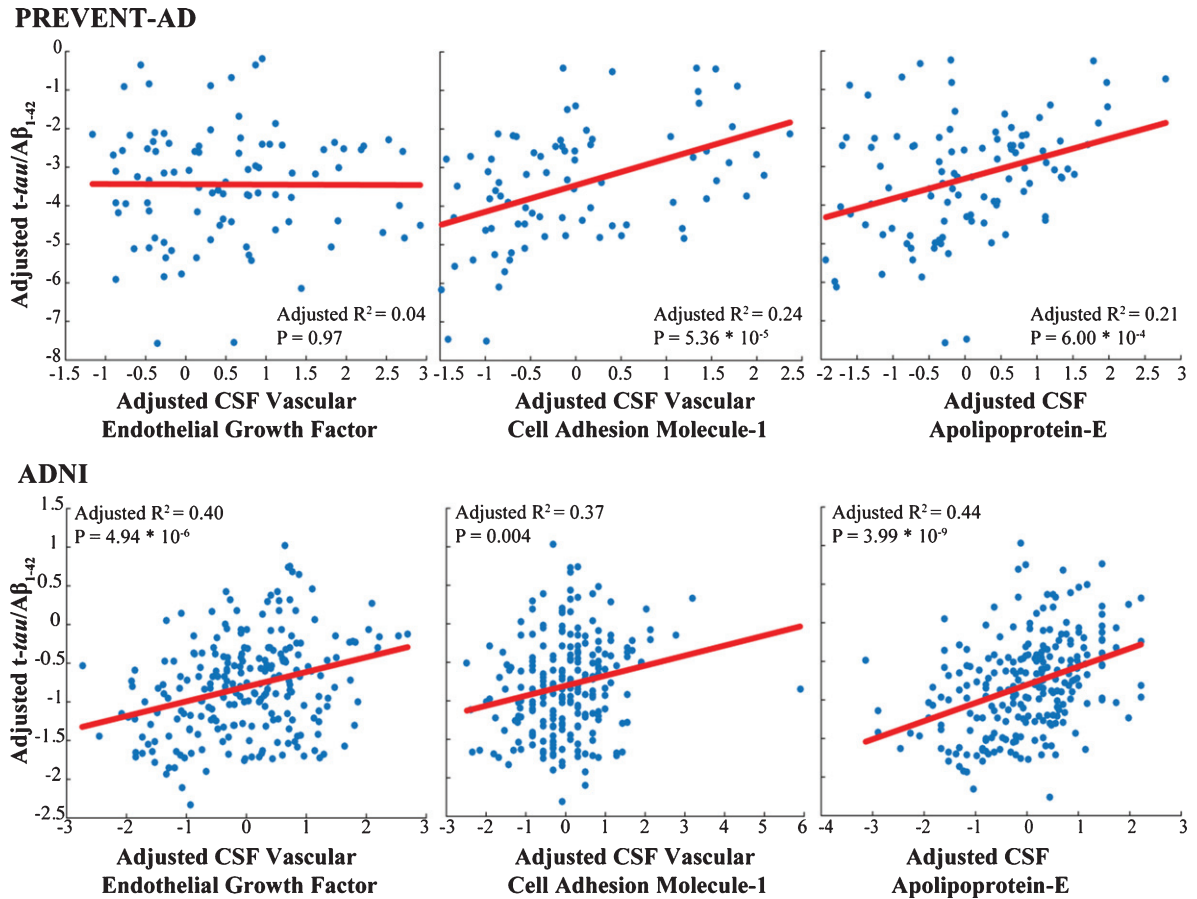
PREVENT-AD

ADNI

Fig. 3. Markers common to both cohorts show similar association with AD pathology. Adjusted response plots show the association of apolipoprotein E level, VEGF and VCAM-1 with t-*tau*/Aβ_{1-42} ratio. R^2 values are given for the fully adjusted model, and *p*-values are reported for the given CSF marker in this model.

Table 3
Coefficients of fully adjusted linear regression models evaluating the association of CSF markers with pathological stage

	Age (y)	Gender	*APOE ε*4 carrier status	Clinical Diagnostic Group	Pathological Stage
PREVENT-AD					
apoE	β: 0.00 p: 0.29	β: 0.08 p: 0.71	β: –0.08 p: 0.74		**β: 0.32 p: 0.002**
VCAM-1	β: 0.00 p: 0.20	β: 0.15 p: 0.58	β: –0.28 p: 0.29		**β: 0.40 p: 0.002**
VEGF	β: 0.00 p: 0.23	β: –0.27 p: 0.29	β: –0.16 p: 0.56		β: 0.03 p: 0.77
ADNI					
apoE	β: 0.01 p: 0.22	**β: –0.39 p: 0.002**	**β: –0.47 p: 0.0006**	β: –0.22 p: 0.10	**β: 0.38 p: 5.38E-7**
VCAM-1	**β: 0.04 p: 5.37E-6**	**β: –0.68 p: 7.08E-8**	β: –0.20 p: 0.12	β: –0.06 p: 0.63	**β: 0.24 p: 7.19E-4**
VEGF	**β: 0.03 p: 1.39E-3**	**β: –0.47 p: 3.26E-4**	β: –0.16 p: 0.24	**β: –0.28 p: 0.04**	**β: 0.29 p: 1.54E-4**

PREVENT-AD: Increased CSF immune activity with advancing AD pathology

Studies in the PREVENT-AD cohort are of particular interest because they focus on changes in the pre-symptomatic or very early-symptomatic evolution of AD pathology. This characteristic of the sample enabled us to examine closely the stages of transition between absence of AD-related changes through early evidence of *tau* pathology. In findings similar to those of others [20], increases in the t-*tau*/Aβ_{1-42} ratio were accompanied by higher concentrations of IL-15, MCP-1, ICAM-1, and VCAM-1. Observations in this cohort that immune markers tended to increase only after the appearance of *tau* abnormality are consistent also with recent

observations in autosomal dominant AD [12]. Furthermore, the pattern is reminiscent of that observed for YKL-40 in pre-clinical "sporadic" AD [14].

ADNI: Bi-directional pattern of association between CSF proteins and pathological stage

Studies in the ADNI sample provided complementary evidence to PREVENT-AD. While ADNI included healthy individuals, these were on average a decade older. In addition, ADNI considered individuals with more severe cognitive symptoms who were accordingly more likely to have advanced pathology. This feature provided a notable opportunity to verify the bi-directional pattern observed in PREVENT-AD. Some 25% of the markers analyzed showed the expected pattern. Interestingly, again most of these proteins also showed a correlation with the t-tau/Aβ_{1-42} ratio.

These results suggest that, independent of clinical diagnosis, there exists a moderate early decrease in CSF immune markers that is not captured by t-tau/Aβ_{1-42} ratio. This pattern appears primarily related to decreasing CSF Aβ_{1-42}. Because, in both samples, CSF t-tau accounts for more variance in the ratio than does Aβ_{1-42}, this effect may ordinarily be hidden by the stronger—opposite– association with tau.

Nature of markers showing the bi-directional pattern

PREVENT-AD markers that exhibit a bi-directional pattern of association with stage are readily recognized as markers of neuro-immune processes. The IL-12/23 signaling pathway is thought to be important in AD, as its regulation may mitigate pathological and cognitive expression of disease in mice [33]. Both IL-12 and IL-23 promote immune responses by stimulating T-cells [34]. IL-12 does so by inducing production of interferon-γ while IL-23 favors the production of IL-17 [35]. It is unclear, however, whether IL-12 levels are elevated in AD [36–38]. IL-8 plays an important role in the chemotaxis of neutrophils [39, 40] which have been recently identified as possible actors in the immune response to AD pathology [41]. IL-8 is a useful marker of intrathecal inflammation and is produced by a variety of activated innate immune cells [42]. Again, however, it is unclear whether it is upregulated in AD [11, 43]. IL-15 is a general stimulator of immunity as it activates natural killer, CD8, and T-cells [44, 45].

Its levels have been reported as being lower in plasma of AD patients [46] but higher in the CSF of AD *vs.* other, non-inflammatory neurological disorders [47].

ICAM and VCAM are cell adhesion molecules. While they play roles in the vascular system, they are also important in immune responses. For instance, endothelial cell VCAM-1 expression is induced by cytokines, promoting adhesion of lymphocytes [48]. In inflammatory reactions, production of VCAM-1 by endothelial cells was initially believed to limit inflammation [49]. Recent work however, suggests that this peptide may play an active role in blood-brain barrier (BBB) disruption [50]. In the immune-activated brain, VCAM is also produced by activated astrocytes [51]. ICAM-1, produced by fibroblast and endothelial cells, is potentiated by IL-1 signaling [52]. ICAM-1 is involved in leukocyte recruitment and migration through the BBB [53]. In all, therefore, these markers appear closely related to the potentiation of innate immunity through recruitment of immune cells via chemotactic signaling or BBB disruption.

Although they are individually different, ADNI markers showing the same bi-directional relation to pathological stage appear to have overlapping mechanisms of action (Fig. 4). For instance, AXL receptor tyrosine kinase [54, 55], IL-3 [56], CD40 antigen [57], Macrophage colony stimulating factor [58], Lectin-like oxidized LDL receptor [59], and Chromogranin-A [60] among others have been implicated in activation and recruitment of immune cells in CNS inflammatory processes. Similarly, other markers such as VEGF [61], VCAM-1 and the Matrix-Metalloproteinase 3 [62] are associated with vascular function but may facilitate the entrance of immune cells into the CNS through either chemotaxis or BBB breakdown. All of this suggests that several immune and vascular changes may occur concurrently with the appearance of AD pathology, as suggested previously [3].

It is unclear why most of these markers remain unchanged or are diminished with appearance of early amyloid pathology. Speculative explanations include a lack of inflammatory response to initial AD pathology [12, 13, 15]. However, animal work has shown that Aβ plaques are surrounded by activated microglia with associated cytokine production (reviewed by [4]). An interesting observation in both cohorts is that, despite their small numbers, participants with 'SNAP' have even higher immune marker levels than the Stage 2 group. This observation is relatively weak in PREVENT-AD, where it reaches
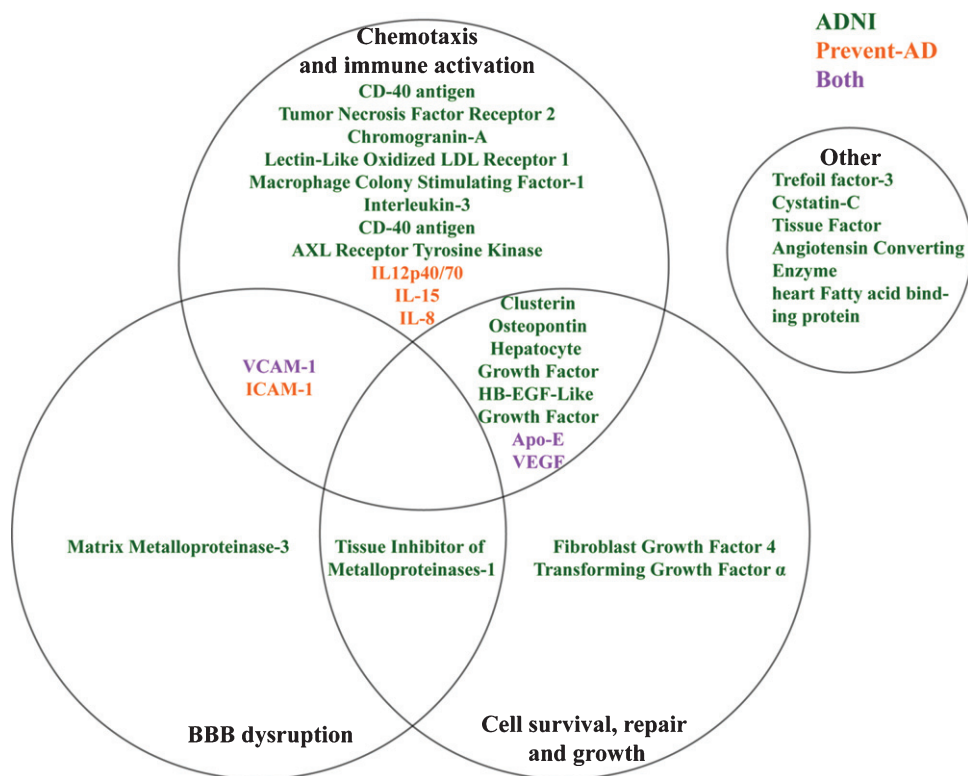
**Chemotaxis
and immune activation**
CD-40 antigen
Tumor Necrosis Factor Receptor 2
Chromogranin-A
Lectin-Like Oxidized LDL Receptor 1
Macrophage Colony Stimulating Factor-1
Interleukin-3
CD-40 antigen
AXL Receptor Tyrosine Kinase
IL12p40/70
IL-15
IL-8

ADNI
Prevent-AD
Both

Other
Trefoil factor-3
Cystatin-C
Tissue Factor
Angiotensin Converting
Enzyme
heart Fatty acid bind-
ing protein

Clusterin
Osteopontin
Hepatocyte
Growth Factor
HB-EGF-Like
Growth Factor
Apo-E
VEGE

VCAM-1
ICAM-1

Matrix Metalloproteinase-3

Tissue Inhibitor of
Metalloproteinases-1

Fibroblast Growth Factor 4
Transforming Growth Factor α

BBB dysruption

**Cell survival, repair
and growth**

Fig. 4. Overlapping theoretical biological pathways of identified ADNI and PREVENT-AD proteins.

statistical significance only for IL12-P70 ($p = 0.001$), but is stronger in ADNI where it holds for 17/23 proteins (data not shown, all $p < 0.05$). These findings raise the possibility that Aβ plaques initially buffer or diminish the normal brain immune response to insult. Additional studies, including longitudinal observations will be necessary to evaluate this idea.

Strengths and limitations

A clear limitation in PREVENT-AD is its small numbers of individuals at pathological Stages 1 and, especially, 2. Another caveat is that only VCAM-1 remained significantly reduced at Stage 1 or increased at Stage 2 when we included the 19 participants with AD biomarkers in the ± 5% "no man's land" around inter-stage thresholds. Even so, an association with stage for the remaining four immune markers remained evident at a trend level of $P < 0.1$ (unadjusted). Furthermore, the differences in PREVENT-AD and ADNI assays for measurement of CSF AD and protein markers pose some difficulty for the interpretation of these results. In all, three markers assayed in both cohorts showed

similar results, thereby supporting our conclusions. At the very least, therefore, studies of immune mechanisms in AD should consider stage and extent of pathological change beyond clinical diagnosis.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-170887.

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