

RESEARCH ARTICLE

Key inflammatory pathway activations in the MCI stage of Alzheimer's disease

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Abstract

Objective: To determine the key inflammatory pathways that are activated in the peripheral and CNS compartments at the mild cognitive impairment (MCI) stage of Alzheimer's disease (AD). **Methods:** A cross-sectional study of patients with clinical and biomarker characteristics consistent with MCI-AD in a discovery cohort, with replication in the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort. Inflammatory analytes were measured in the CSF and plasma with the same validated multiplex analyte platform in both cohorts and correlated with AD biomarkers (CSF A β 42, total tau (t-tau), phosphorylated tau (p-tau) to identify key inflammatory pathway activations. The pathways were additionally validated by evaluating genes related to all analytes in coexpression networks of brain tissue transcriptome from an autopsy confirmed AD cohort to interrogate if the same pathway activations were conserved in the brain tissue gene modules. **Results:** Analytes of the tumor necrosis factor (TNF) signaling pathway (KEGG ID:4668) in the CSF and plasma best correlated with CSF t-tau and p-tau levels, and analytes of the complement and coagulation pathway (KEGG ID:4610) best correlated with CSF A β 42 levels. The top inflammatory signaling pathways of significance were conserved in the peripheral and the CNS compartments. They were also confirmed to be enriched in AD brain transcriptome gene clusters. **Interpretation:** A cell-protective rather than a proinflammatory analyte profile predominates in the CSF in relation to neurodegeneration markers among MCI-AD patients. Analytes from the TNF signaling and the complement and coagulation pathways are relevant in evaluating disease severity at the MCI stage of AD.

Introduction

Alzheimer's disease (AD) is characterized by a progressive decline in cognitive function and is the most common cause of dementia worldwide. Even as classical AD neuropathology is described as amyloid plaques and neurofibrillary tangles in the brain, it is recognized that a complex cascade of events involving additional molecular players likely play a crucial role in the disease onset and progression.

Accumulating evidence implicates inflammatory changes in AD.^{1–3} The role of systemic inflammation and occurrence of amyloid plaque-dependent inflammation have been well documented in both human autopsy specimens and animal models.^{4,5} Altered expression of multiple cytokines and chemokines in AD patients compared to controls have been reported.^{3,6–10} Alterations in systemic inflammation, including plasma levels of tumor necrosis factor (TNF)- α -related cytokines have been linked to worsened cognition in AD.^{3,10,11} In addition, genome-wide association studies have demonstrated that polymorphisms in several inflammatory genes are associated with modestly increased risk for AD.^{12,13} A role for inflammation and immune mechanisms in AD therefore appears highly likely.^{14,15}

In spite of recognition of the role for inflammation in the pathophysiology of AD, there are some key outstanding questions. (1) Which key inflammatory pathways are activated in clinical AD? Among most prior studies of inflammatory changes in AD patients, small number of inflammatory analytes were measured, typically 10 or fewer.^{6,10,11,16–18} To help identify inflammatory pathways or networks of inflammatory analytes pertinent to AD and to evaluate if they are helpful or harmful to cognition, it is essential to develop approaches that evaluate multiple analytes concomitantly and interrogate their biological significance when expressed together, (2) Prior studies have predominantly focused on the peripheral compartment and it remains unclear how the inflammatory changes in the CNS relates to changes in the peripheral compartment for a large set of inflammatory molecules. This knowledge is critical to a mechanistic understanding of the pathophysiological changes related to inflammation in the peripheral and CNS compartments simultaneously and to potentially evaluate the utility of monitoring these inflammatory changes in AD for clinical outcomes.

We therefore took a systematic approach to answer these two questions among mild cognitive impairment (MCI)-AD clinical patients. After evaluation in our discovery cohort we validated the results among MCI patients in the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort. We also determined if the genes related to soluble inflammatory analytes evaluated in our

discovery cohort have also been transcribed in the brain tissue, using transcriptomic data from autopsied AD brains. Using functional network enrichment analysis we determined the biological pathways most likely related to the inflammatory analytes and genes of significance and its potential convergence across multiple datasets of brain, CSF, and plasma in the presence of markers of AD pathology.

Materials and Methods

Discovery cohort

A cross-sectional discovery study of 48 MCI-AD patients was conducted. All patient consent was obtained according to the Declaration of Helsinki and the study was approved by the Cleveland Clinic Institutional Review Board. The patients were recruited from a specialized memory clinic. The diagnosis of MCI-AD was confirmed by the presence of CSF A β 42 and p-tau levels consistent with a diagnosis of AD and consensus evaluation of two neurologists and a neuropsychologist by published criteria.¹⁹ A commercially available test from Athena Diagnostics, ADmark® Alzheimer's evaluation Innotech sandwich Enzyme Linked Immunosorbant Assay (ELISA) kits was used for measuring CSF levels of A β 42, t-tau, and p-tau levels. The subjects met cut offs CSF A β 42 \leq 530pg/mL, CSF p-tau \geq 60pg/mL which are consistent with Amyloid positive status on the Amyvid™ PET at our center. An additional marker of neuronal degeneration in the CSF, neuron-specific enolase (NSE), was measured in the rules-based-medicine (RBM) multiplex analyte platform described below. NSE is often highly elevated in diseases which result in relative rapid neuronal destruction (hours/days) and levels are therefore supportive of a more recent neuronal loss in relation to analytes measured. APOE status was determined by blood samples (10 ng per subject) dispensed into 96 well plates for TaqMan allelic discrimination detection of single-nucleotide polymorphisms that discriminate the APOE alleles (*rs429358*, *rs7412*) (Life Technologies). PCR reactions were carried out using a 9700 Gene Amp PCR system (Applied Biosystems, CA) and an end-point read in a 7500 Real-Time PCR system (Applied Biosystems, CA). Table 1 provides data on discovery cohort demographics and Figure 1 provides a methodological overview of the study.

Characterizing clinical and environmental factors

Age, sex, education, medical comorbidities family history, smoking, and other addictions and medication use, including nonsteroidal anti-inflammatory drugs (NSAIDs)

Table 1. Demographics and medical history of Discovery and ADNI cohorts.

Factor	Discovery cohort (N = 48)		ADNI cohort (N = 43)		P-value
	n	Statistics	n	Statistics	
Age at enrollment	48	68.1 ± 7.3	43	76.1 ± 6.5	<0.001^a
Gender	48		43		0.11 ^c
Male		28 (58.3)		32 (74.4)	
Female		20 (41.7)		11 (25.6)	
Years of education	48	16.0 [12.5, 18.0]	43	16.0 [15.0, 18.0]	0.12 ^b
APOEε4 positive	48	37 (77.1)	43	21 (48.8)	0.005^c
MMSE	48	24.8 ± 3.1	43	26.6 ± 1.6	<0.001^a
CDR-SB	48	2.2 ± 1.3	43	1.5 ± 0.79	0.003^a
DRS	47	125.6 ± 10.3		NA	
CSF Aβ42, pg/mL	48	305.9 [216.1, 367.1]	43	144.0 [135.0, 167.0]	<0.001^b
CSF t-tau, pg/mL	48	454.3 [335.1, 771.]	43	91.0 [64.0, 132.0]	<0.001^b
CSF p-tau, pg/mL	48	79.6 [59.3, 104.6]	43	33.0 [21.0, 42.0]	<0.001^b

Statistics presented as Mean ± SD, Median [P25, P75] or N (column %).

P-values: a = ANOVA, b = Kruskal–Wallis test, c = Pearson’s chi-square test.

MMSE, Mini-mental state exam; CDR-SB, Clinical dementia rating scale-sum of boxes.

DRS, Dementia rating scale. P values in bold meet statistical significance <0.05.

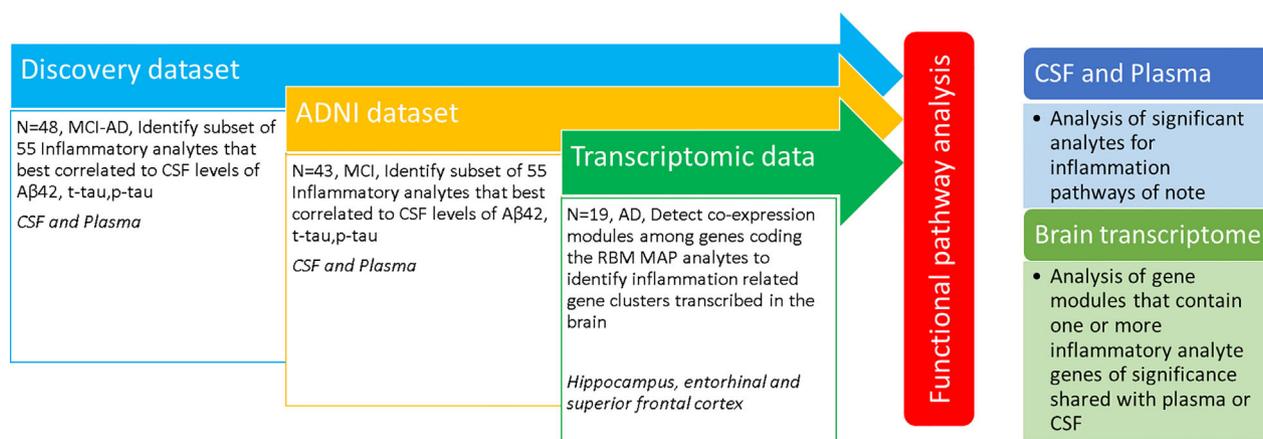


Figure 1. Methodological overview.

were characterized. Confounding treatment with steroids, trauma, and surgical intervention and infectious disease in the last 6 months, in addition to uncontrolled diabetes and vascular disease as part of clinical evaluation were screened. Additional preexisting proinflammatory disorders (e.g., systemic lupus erythematosus) were recorded and levels of C-reactive protein (CRP) and sedimentation rate related to systemic inflammation was quantified in the blood. Table S1.

Neuropsychological tests

Mini-Mental State Examination (MMSE),²⁰ WMS-IV Logical Memory 1 and 2,²¹ Clinical Dementia Rating scale (CDR-SB and Global),²² and Dementia Rating Scale

(DRS)²³ tests were conducted to characterize the degree of their baseline cognitive and functional deficits.

Inflammatory biomarkers

CSF and plasma were collected and analyzed by an independent laboratory via the validated RBM Multi-Analyte Profile (MAP) platform from Myriad Genetics (Salt Lake City, UT). Samples were evaluated for levels of 86 analytes using a custom MAP: HumanMAP® v2.0 + IL1 and 16 using a Luminex platform. Validation has been performed as defined by the Clinical and Laboratory Standards Institute and is therefore replicable across multiple runs. CSF and plasma samples were collected contemporaneously. They were both frozen within 15 min after

collection and processed (at -70°C in dry ice) and maintained at a freezing temperature (-80°C at a maximum nonfrost-free type refrigerator) continuously. The samples were shipped frozen, in a Styrofoam container with sufficient dry ice to maintain temperature less than -70°C for at least 48 h. Samples therefore underwent a single freeze thaw cycle prior to analyses. Each analyte measure had to pass a threshold of measurement and quality control criteria set by Myriad Genetics.

The consistency of the data was further evaluated by internal and external validation. There were two technical replicates of CSF and plasma samples chosen randomly from three patients to evaluate consistency of data. Expected high degree of correlation within individual subjects analyte profiles between markers of positive acute phase response (CRP, haptoglobin, ferritin, $\alpha 2$ macroglobulin, fibrinogen) was further confirmed and the results from the discovery cohort were next evaluated in ADNI.

ADNI validation cohort

ADNI is a longitudinal multicenter study designed to develop clinical, imaging, genetic, and biochemical biomarkers for the early detection and tracking of AD. ADNI was launched by the National Institute of Aging and is a multicenter project with additional support from private pharmaceutical companies, and nonprofit organizations. ADNI 1 eligibility criteria are described in the ADNI 1 protocol <http://adni.loni.usc.edu/methods/documents/>. Briefly, participants are 55–90 years of age, had an informant able to provide an independent evaluation of functioning, and spoke either English or Spanish. Participants had completed at least 6 years of education (or had a work history sufficient to exclude mental retardation). General inclusion/exclusion criteria for MCI subjects are as follows: MMSE scores between 24 and 30 (inclusive; exceptions made on a case by case basis by neurologists at the center of follow up), a memory complaint, objective memory loss measured by education adjusted scores on Wechsler Memory Scale Logical Memory II, a CDR of 0.5, absence of significant levels of impairment in other cognitive domains, essentially preserved activities of daily living, and an absence of dementia.

The demographics at baseline among the subset of all 43 ADNI MCI participants who had CSF and plasma multiplex data were used in the validation analysis are shown in Table 1. These subjects were noted to have CSF $A\beta 42$, t-tau, and p-tau levels supportive of underlying AD pathology.²⁴ Details on the Luminex method used for ADNI AD biomarkers measurement are detailed elsewhere.²⁵ CSF samples were measured for levels of 159 analytes using the RBM DiscoveryMAP® v.1.0 panel. The RBM HumanMAP® v.2.0 used in the discovery cohort is a subset of the RBM DiscoveryMAP® v.1.0 and uses a

Luminex platform with the same quality control and thresholding process used in ADNI dataset and are comparable. We analyzed data from ADNI's well-characterized subset of MCI participants who had curated CSF and plasma multiplex data, the details of which have been published previously.²⁶ The data from CSF QC multiplex data used in this analysis is the cleaned, quality controlled data according to methodology described in the statistical analysis of Biomarkers Consortium data primer.²⁷

Bioinformatics and statistical analysis

Ontology analysis and network analysis to identify inflammatory analytes

Gene/protein annotations and gene ontologies were downloaded from the Gene Ontology (GO)²⁸ and processed into tabular files containing GO terms annotated to genes and the gene ontology tree. The RBM analyte names were mapped to HUGO Gene Nomenclature Committee gene symbols by manual curation. All analytes had a one to one correspondence with coding genes with the exceptions of ferritin (heavy chain coding gene *FTH1*), and fibrinogen (alpha chain coding gene *FGA*). The full ontology tree rooted at the inflammatory response node (GO:0006954) was constructed and all genes labeled by this term were extracted ($n = 458$). The analytes identified in the proprietary RBM InflammationMAP® v1.0 ($n = 45$ analytes) and the inflammatory response genes from GO analysis were combined ($n = 483$) and used in a network analysis to identify additional analytes from the RBM HumanMAP® v2.0 that are related to inflammation. The Crosstalker algorithm was run using the 483 seeds to rank the RBM HumanMAP® by their proximity to the seeds.^{29,30} The analysis was run using three reference networks (STRINGv10.5, BioGRIDv3.4.163, and Human Protein Reference Database, all latest versions), and repeated three times on each network since the algorithm is nondeterministic.^{21,31} For a target analyte to be deemed significant it had to pass the significance threshold in all three runs for the same reference network where significance was set at $P < 0.2$ ($z > 0.85$). This identified an additional eight analytes from the RBM HumanMAP® that were combined with the RBM InflammationMAP® ($n = 45$ analytes) to form our final comprehensive list of 53 candidate inflammatory analytes. The list of inflammatory analytes analyzed in the study is provided in Table 2.

Statistical analysis

The CSF and plasma analytes of interest identified above were log transformed, which limits the impact of extreme measures. Only those with at least 50% response rate of

analytes above the limit of detection were included for further analysis Figure S1. The absolute values of the inflammatory markers and AD biomarkers were compared. Normality of biomarkers was evaluated using graphical methods and the Shapiro–Wilk test and a log (base 2) transformation allowed Pearson correlations to be fit. Along with estimates of correlation, 95% confidence intervals and *P*-values with and without false discovery rate (FDR) adjustment were calculated. If we assume 53 analytes (corresponding to a Bonferroni adjusted significance level of 0.009), with 48 CSF samples and 46 plasma samples used in each analysis, there would be 80% power to detect correlations above 0.5 as statistically significant. Analysis was performed unadjusted and then adjusting for age, sex, baseline MMSE, and *APOEε4* status (present vs. absent). Agreement on relative ordering of response of analytes between the discovery and ADNI data was evaluated using Kendall's *W* coefficient of concordance statistic. 95% confidence intervals for kappa statistics and *P*-values testing whether more agreement than expected by chance were calculated for both measures. Analysis was performed using SAS software (version 9.4) and an overall significance level of 0.05 was assumed for all tests.

Subgroup searching for analyte synergistic relationships

In order to evaluate analyte levels that show higher correlation when considered together (synergistic relationship) rather than individual analyte correlation by univariate analysis alone, we performed an exhaustive search to find analyte subgroups whose aggregate levels maximally correlated with AD CSF biomarkers. The full panel of analytes was filtered to contain only those which were detected in at least 50% of the data. All subgroups of analytes were enumerated up to maximum group size of six analytes. Aggregate activity of each subgroup was computed by first normalizing each analyte by subtracting the mean and dividing by the standard deviation, and then summing normalized analyte levels for each patient.^{32,33} The Pearson correlation coefficient was computed between the aggregate activity of each analyte group and all response markers (e.g., t-tau levels). Only the highest scoring analyte subgroup was reported for each response marker in CSF and plasma, respectively. Significance *P*-values for findings were estimated by two tests using an approach that has been previously described.³² Hypothesis 1(H1) tested how likely we were to see greater or equal correlation with random analyte subgroups by sampling 10,000 random analyte subgroups (of the same size as each actual best scoring analyte subgroup) from among all analytes that met the 50% detection threshold (i.e., not just the inflammatory analytes) and computing the

correlation values. *P*-values were estimated as the fraction of random subgroups that achieved greater or equal correlation with the response marker as the actual analyte subgroup. Hypothesis 2(H2) tested how likely we were to randomly observe greater or equal correlation between the aggregate activity of an analyte subgroup and a response marker by permuting the values of each response marker 10,000 times and computing the correlation values to the aggregate analyte levels. *P*-values were estimated as the proportion of randomized responses with equal or greater correlation with aggregate analyte levels than the actual response.

Functional pathway analysis on analytes of interest

The analytes of significance identified in the univariate statistical analysis and confirmed in the analyte subgroup search above in relation to CSF neurodegeneration markers were entered into STRING: functional protein association networks for pathway enrichment analysis.³⁴ The top pathway for each analysis with the largest gene count and the lowest *P* value following FDR correction is reported.

Weighted gene coexpression network analysis (WGCNA) in autopsy AD brains

The R package WGCNA³⁵ was used to construct coexpression networks as in prior studies³⁶ using Alzheimer's Disease Dataset: GSE 48350 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48350>). Here, postmortem brain tissue had been collected from ADRC brain banks, data from all 19 AD brains in the dataset with hippocampal, entorhinal cortex, and superior frontal cortex transcriptomic data were used for the current analysis. The GSE48350 expression data were reduced to probe sets corresponding to the 84 RBM full panel genes. Four HumanMAP® RBM analytes were not represented by any probe sets (IL12, IgE, Chorionic Gonadotropin Subunit Beta 3, and CA-19-9). The remaining 80 RBM analytes were represented by 198 probe sets. The modules with one or more inflammatory analyte genes of significance shared with plasma or CSF were characterized using GO Elite to control the network-wide false discovery rate, with all enriched pathways comprising at least 10 genes at $Z > 2$ and $FDR < 0.01$.³⁷ The gene modules were characterized for their functional enrichment of biological pathways using STRING³⁴ to evaluate their functional role.

Results

Subject demographics of the discovery and ADNI cohorts are in Table 1. Summary statistics of the 53 inflammatory

Table 2. List of inflammatory analytes analyzed.

RBM Name	Gene	RBM Name	Gene
1. Alpha-1-Antitrypsin	SERPINA1	27. Interleukin-12 Subunit p40	IL12B
2. Alpha-2-Macroglobulin	A2M	28. Interleukin-12 Subunit p70	IL12P70
3. Apolipoprotein A-I	APOA1	29. Interleukin-15	IL15
4. Beta-2-Microglobulin	B2M	30. Interleukin-17	IL17A
5. Brain-Derived Neurotrophic Factor	BDNF	31. Interleukin-18	IL18
6. Complement C3	C3	32. Interleukin-8	CXCL8
7. C-Reactive Protein	CRP	33. Interleukin-23	IL23A
8. Eotaxin-1	CCL11	34. Macrophage Inflammatory Protein-1 alpha	CCL3
9. Fibrinogen	FGA	35. Macrophage Inflammatory Protein-1 beta	CCL4
10. Factor VII	F7	36. Matrix Metalloproteinase-3	MMP3
11. Ferritin	FTH1	37. Matrix Metalloproteinase-9	MMP9
12. Granulocyte-Macrophage Colony-Stimulating Factor	CSF2	38. Monocyte Chemotactic Protein 1	CCL2
13. Granulocyte Colony-Stimulating Factor	CSF3	39. Matrix Metalloproteinase-2	MMP2
14. Haptoglobin	HP	40. Myeloperoxidase	MPO
15. Intercellular Adhesion Molecule 1	ICAM1	41. Neuron-Specific Enolase	ENO2
16. Interferon gamma	IFNG	42. Plasminogen Activator Inhibitor 1	SERPINE1
17. Interleukin-1 alpha	IL1A	43. Serotransferrin	TF
18. Interleukin-1 beta	IL1B	44. Stem Cell Factor	SCF
19. Interleukin-1 receptor antagonist	IL1RN	45. T-Cell-Specific Protein RANTES	CCL5
20. Interleukin-2	IL2	46. Tissue Inhibitor of Metalloproteinases 1	TIMP1
21. Interleukin-3	IL3	47. Tumor Necrosis Factor alpha	TNF
22. Interleukin-4	IL4	48. Tumor Necrosis Factor beta	LTA
23. Interleukin-5	IL5	49. Tumor Necrosis Factor Receptor 2	TNFRSF1B
24. Interleukin-6	IL6	50. Vascular Cell Adhesion Molecule-1	VCAM1
25. Interleukin-7	IL7	51. Vascular Endothelial Growth Factor	VEGFA
26. Interleukin-10	IL10	52. Vitamin D-Binding Protein	GC
		53. von Willebrand Factor	VWF

analytes evaluated in CSF and plasma are provided in Table S2.

Discovery cohort

Univariate analysis CSF

In the unadjusted analyses, after applying the FDR correction, TNFR2, SCF, Ferritin, and α 2macroglobulin were positively correlated with CSF t-tau, p-tau, and NSE. After adjusting for covariates (age, sex, baseline MMSE, *APOE* ϵ 4 status), the same associations were significant. In addition, MMP2, MMP3, β 2 microglobulin, VCAM1, and VEGF were positively correlated with CSF t-tau, and p-tau but not NSE. After adjustment for the same covariates in addition to the above five analytes, CCL2 (MCP1) and vWF were also found to be significantly correlated with CSF t-tau and p-tau. (Table 3). There were no significant correlations of the CSF inflammatory analytes to CSF *A* β 42.

Univariate analysis plasma

In unadjusted analysis after applying the FDR correction, only α 2-macroglobulin was positively correlated with CSF

t-tau and p-tau measures and MMP9 was positively correlated with CSF NSE. After adjustment for covariates, the associations between α 2-macroglobulin and CSF p-tau and between MMP9 and CSF NSE remained significant (Table 4). There were no significant correlations of the plasma inflammatory analytes to CSF *A* β 42.

Secondary analyses

None of the CSF and plasma inflammatory analytes had a significant degree of correlation with any of the cognitive measures after applying the FDR correction. No difference was noted in the analytes of significance when adjusted for NSAID exposure. Adjusting for individual CSF/plasma albumin ratio noted no decrease in the number of analytes of significance on the Pearson correlations corrected for FDR. While, complement C3 and vWF were the additional analytes from the unadjusted correlations with CSF t-tau now meeting FDR correction.

When comparing univariate correlations between plasma and CSF inflammatory analytes and CSF AD biomarkers in the discovery cohort, the correlation values to AD biomarkers for plasma inflammatory analytes were lower than those for CSF (Tables 3 and 4 and

Table 3. Significant associations between CSF inflammatory analytes and CSF neurodegenerative markers (adjusted for age, sex, APOEε4, and MMSE) in discovery cohort.

Factor 1	Factor 2	N	Pearson r (95% CI)	Raw P-value	False discovery rate (FDR) P-value
Aβ42	VDBP	47	0.37 (0.08, 0.60)	0.014	0.16
Total Tau	TNFR2	48	0.73 (0.55, 0.84)	<0.001	<0.001
	SCF	48	0.66 (0.44, 0.80)	<0.001	<0.001
	B2M	47	0.62 (0.38, 0.77)	<0.001	<0.001
	Ferritin	48	0.58 (0.33, 0.74)	<0.001	<0.001
	A2M	48	0.50 (0.24, 0.69)	<0.001	0.002
	MMP3	48	0.49 (0.22, 0.69)	<0.001	0.002
	VCAM1	48	0.46 (0.18, 0.66)	0.002	0.012
	VEGF	48	0.42 (0.14, 0.64)	0.004	0.012
	CCL2 (MCP1)	48	0.38 (0.09, 0.61)	0.009	0.023
	vWF	48	0.34 (0.05, 0.58)	0.022	0.047
PhosphoTau	TNFR2	48	0.72 (0.54, 0.84)	<0.001	<0.001
	SCF	48	0.71 (0.52, 0.83)	<0.001	<0.001
	B2M	47	0.60 (0.36, 0.76)	<0.001	<0.001
	Ferritin	48	0.59 (0.35, 0.75)	<0.001	<0.001
	A2M	48	0.57 (0.32, 0.74)	<0.001	0.002
	VCAM1	48	0.48 (0.21, 0.68)	<0.001	0.003
	MMP3	48	0.46 (0.18, 0.66)	0.002	0.006
	VEGF	48	0.43 (0.14, 0.64)	0.003	0.009
	vWF	48	0.41 (0.12, 0.63)	0.005	0.013
	CCL2 (MCP1)	48	0.35 (0.05, 0.58)	0.020	0.045
NSE	Ferritin	48	0.75 (0.58, 0.85)	<0.001	<0.001
	SCF	48	0.71 (0.51, 0.83)	<0.001	<0.001
	TNFR2	48	0.68 (0.47, 0.81)	<0.001	<0.001
	CCL4 (M1P1β)	48	0.48 (0.21, 0.68)	<0.001	0.004
	vWF	48	0.48 (0.21, 0.68)	<0.001	0.005
	A2M	48	0.46 (0.18, 0.66)	0.002	0.008
	MMP2	47	0.39 (0.10, 0.62)	0.009	0.036
	VCAM1	48	0.36 (0.06, 0.59)	0.017	0.045
	MMP3	48	0.31 (0.01, 0.55)	0.043	0.10
	AAT	47	-0.36 (-0.60, -0.07)	0.016	0.045

FDR P values in bold meet statistical significance < 0.05.

Table 4. Significant associations between Plasma inflammatory analytes and CSF neurodegenerative markers (adjusted for age, sex, APOEε4, and MMSE) in discovery cohort.

Factor 1	Factor 2	N	Pearson r (95% CI)	Unadjusted P-value	False discovery rate (FDR) P-value
ABeta42	CCL4	46	0.39 (0.09, 0.61)	0.011	0.35
	AAT	46	0.31 (0.01, 0.56)	0.042	0.41
Total Tau	A2M	46	0.38 (0.08, 0.61)	0.013	0.20
	ICAM1	45	-0.40 (-0.63, -0.10)	0.009	0.20
PhosphoTau	A2M	46	0.49 (0.22, 0.69)	<0.001	0.023
	ICAM1	45	-0.36 (-0.60, -0.05)	0.020	0.31
	VEGF	45	-0.43 (-0.65, -0.13)	0.005	0.10
NSE	MMP9	45	0.57 (0.31, 0.74)	<0.001	0.002
	Haptoglobin	46	-0.39 (-0.62, -0.09)	0.010	0.16

FDR P values in bold meet statistical significance < 0.05.

Fig. S2). On Synergistic group analysis A2M, CCL2, ENO2, MMP3, and TNFRSF1B shared significance between plasma and CSF t-tau, whereas CCL4, HP, and SERPINA1 shared synergistic correlations with Aβ42 (Table 5).

Comparing inflammatory analytes in ADNI versus the discovery cohort

On univariate comparisons none of the inflammatory analyte correlations with CSF neurodegeneration markers

Table 5. Analyte subgroup analysis with most significant inflammatory analytes in discovery cohort.

CSF factor (+correlation, -correlation)	<i>r</i>	H1 <i>P</i> -value	H2 <i>P</i> -value	Analyte subgroup	Shared with plasma	Shared with ADNI dataset
Aβ42+	0.69	<0.0001	<0.0001	SERPINA1,CRP,HP,CCL4,ENO2,CCL5, VEGFA,GC,VWF	CCL4,HP, SERPINA1	
Aβ42-	-0.039	0.074	0.40	SERPINE1		
t-tau+	0.917	<0.0001	<0.0001	A2M,CRP,F7,FTH1,MMP3,CCL2, ENO2,TNFRSF1B,VWF	A2M,CCL2,ENO2, MMP3,TNFRSF1B	FTH1,MMP3
t-tau -	-0.148	0.018	0.14	SERPINA1,CRP		CRP
p-tau+	0.963	<0.0001	<0.0001	A2M,F7,FTH1,MMP3,CCL2,ENO2, TNFRSF1B,VWF	A2M,CCL2,ENO2, MMP3	FTH1,MMP3
PTAU-	-0.137	0.069	0.17	SERPINA1		
Plasma factor (+correlation, -correlation)	<i>r</i>	H1 <i>P</i> -value	H2 <i>P</i> -value	Analyte subgroup	Shared with CSF	Shared with ADNI dataset
Aβ42+	0.745	<0.0001	<0.0001	SERPINA1,BDNF,F7,FTH1,HP,CCL4,MMP3	CCL4,HP, SERPINA1	FTH1,HP
Aβ42-	-0.795	<0.0001	<0.0001	C3,IL1RN,IL18,MMP2,MMP9,ENO2,CCL5, GC		MMP2,MMP9
t-tau+	0.64	<0.0001	0.0011	A2M,B2M,IL12B,MMP3,CCL2,ENO2, TNFRSF1B,GC	A2M,CCL2,ENO2, MMP3,TNFRSF1B	
t-tau -	-0.735	<0.0001	<0.0001	B2M,CCL11,ICAM1,MMP9,SERPINE1, KITLG,CCL5,VCAM1,VEGFA,GC		B2M,CCL11, MMP9,SERPINE1
p-tau+	0.685	<0.0001	0.0003	A2M,B2M,IL12B,CCL4,MMP2, MMP3, CCL2,ENO2,TIMP1,GC	A2M,CCL2,ENO2, MMP3	CCL4,MMP2
p-tau -	-0.71	<0.0001	<0.0001	B2M,CCL11,ICAM1,MMP9,SERPINE1, KITLG,CCL5,VCAM1,VEGFA,GC		

H1 and H2 *P* values in bold meet statistical significance < 0.05.

within the ADNI dataset met $P < 0.05$ FDR correction threshold. However, most inflammatory analytes shared similar direction and similar relative ordering/ranking of correlation distances between ADNI and discovery data (Fig. 2). Comparing ADNI and the discovery cohorts, the ordering of the correlations between CSF inflammatory analytes and neurodegeneration markers was significantly higher than chance in relation to CSF t-tau and p-tau (mean diff in correlation: 0.32, *W* statistic 0.92, $P = 0.013$ for t-tau). On average, the correlations between the cohorts were 0.3 or higher in the discovery cohort. The correlation distances between the two cohorts were lower for measures in relation CSF Aβ42 (mean diff in correlation: 0.11, *W* statistic 0.64, $P = 0.19$ for Aβ42) and plasma inflammatory analytes (t-tau, Aβ42: mean diff in correlation: 0.01, 0.03, *W* statistic 0.55, 0.43, $P = 0.33$, 0.68) (Table S3, Fig. S3).

Analyte subgroup analysis for synergistic relationships in discovery and ADNI cohorts

On evaluating analyte levels that show higher correlation when considered together rather than the individual component analytes (synergistic analyte analysis), the results

replicated the significant results of univariate Pearson correlations above. In addition it identified groups of analytes that did not meet statistical significance when considered alone. The analyte subgroups that best correlated with t-tau, p-tau, and NSE had analytes cross represented between them and differed from the analyte subgroup that best correlated with Aβ42 in both the CSF and plasma (Table 5). The synergistic analytes in the discovery cohort that correlated with t-tau that were noted in both plasma and CSF included α-2 Macroglobulin, CCL2, NSE(ENO2), MMP3, and TNFR2 (TNFRS1B). Haptoglobin and α-1-antitrypsin (SERPINA1), while CCL4 was best correlated with Aβ42 in both plasma and CSF synergistic analyte analysis. On comparing ADNI and discovery data subanalyte analysis, they again shared common analyte groups meeting statistical significance that is detailed in Table 5 and Table S4.

Functional pathway analysis

When CSF and plasma analytes that positively correlated with t-tau and p-tau measures from univariate and sub-analyte analyses were entered into STRING,³⁴ the top hit among the Kyoto Encyclopedia of Genes and Genomes

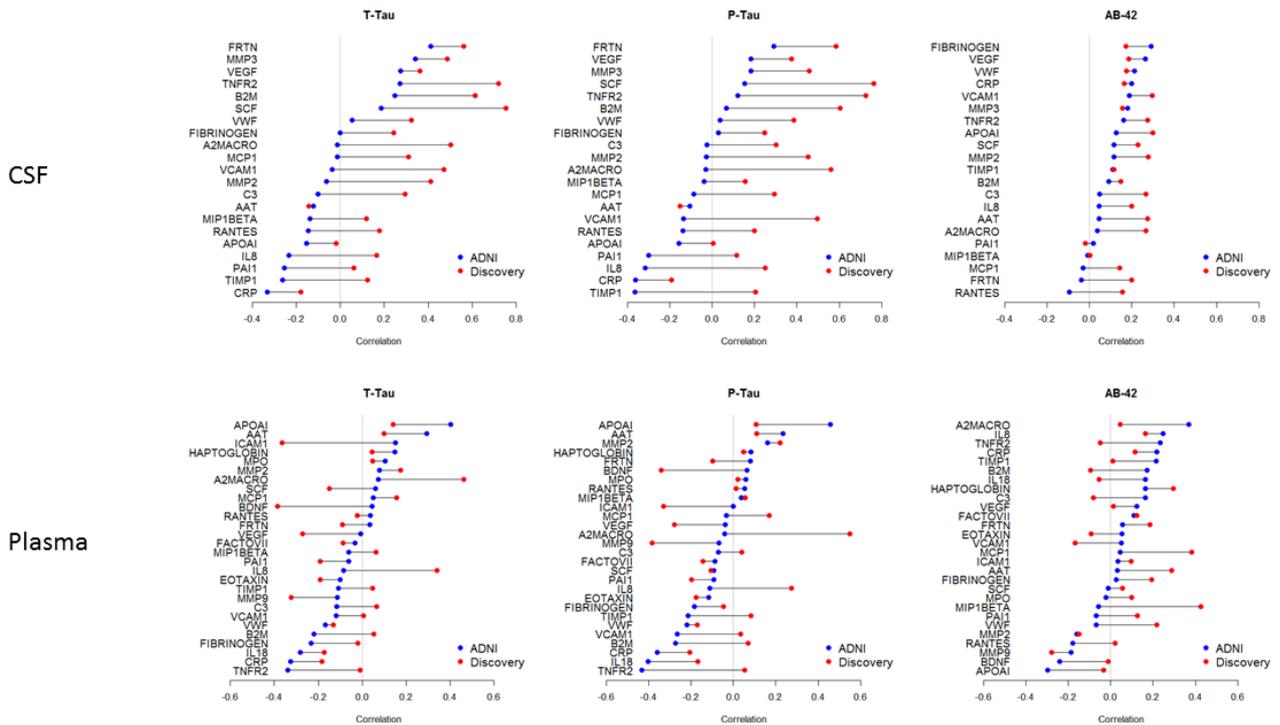


Figure 2. Cluster plot noting similar correlation ordering among the inflammatory analytes in ADNI and discovery cohorts, with shared ordering of analytes more notable in CSF than plasma measures. (Colored points: Pearson correlation coefficients of discovery and ADNI analytes, Connector lines: difference in correlation values for each analyte compared between the two datasets).

(KEGG) pathways³⁸ in both CSF (gene count 4, $P < 0.0001$) and plasma (gene count = 3, $P = 0.0021$) was the TNF signaling pathway (KEGG entry: hsa04668). In relation to A β 42 levels both in the CSF (gene count 3, $P = 0.0012$) and plasma (gene count 3, $P = 0.0022$), the complement and coagulation cascade (KEGG entry: hsa04610) was noted consistently among the top KEGG pathway hits (Table S5).

AD brain transcriptomic analysis

Using autopsy AD brain whole-genome transcriptomic data, three coexpression modules (M1-M3 see Fig. 3 for module details) were identified. The modules represent network gene clusters that share highly similar expression patterns in the three AD brain regions evaluated. Functional analyses by STRING showed that the transcriptomic gene modules again included key inflammatory pathways identified in the CSF and plasma analytes that correlated with AD biomarkers (cytokine–cytokine receptor interaction, complement and coagulation cascade, and TNF signaling pathway) (Fig. S3). The modules share genes with significant analytes from the synergic group analysis (Module 1: A2M, SCF, MMP2, F7, FTH1, CRP, and

AAT. Module 2: TNFRSF1B, B2M, TIMP1, ICAM1, Module 3: did not enrich to a KEGG cluster).

Discussion

This study clearly demonstrates activation of key shared inflammatory pathways across the CSF, plasma and brain tissue in human subjects with AD. In our discovery dataset, the inflammatory analytes that best correlated with levels of t-tau, p-tau, and NSE levels were in TNF signaling pathway and those that best correlated to A β 42 were in the complement and coagulation pathway. These pathway activations were also noted to be enriched in AD brain tissue following a transcriptomic gene coexpression analysis.

Replication of CSF and plasma results in ADNI

The AD biomarkers shared similar relationships to significant inflammatory analytes in both the cohorts (Fig. 2). The discovery cohort with a larger range of AD biomarker values had consistently larger correlation coefficients than ADNI. The median and range of CSF A β 42 and t-

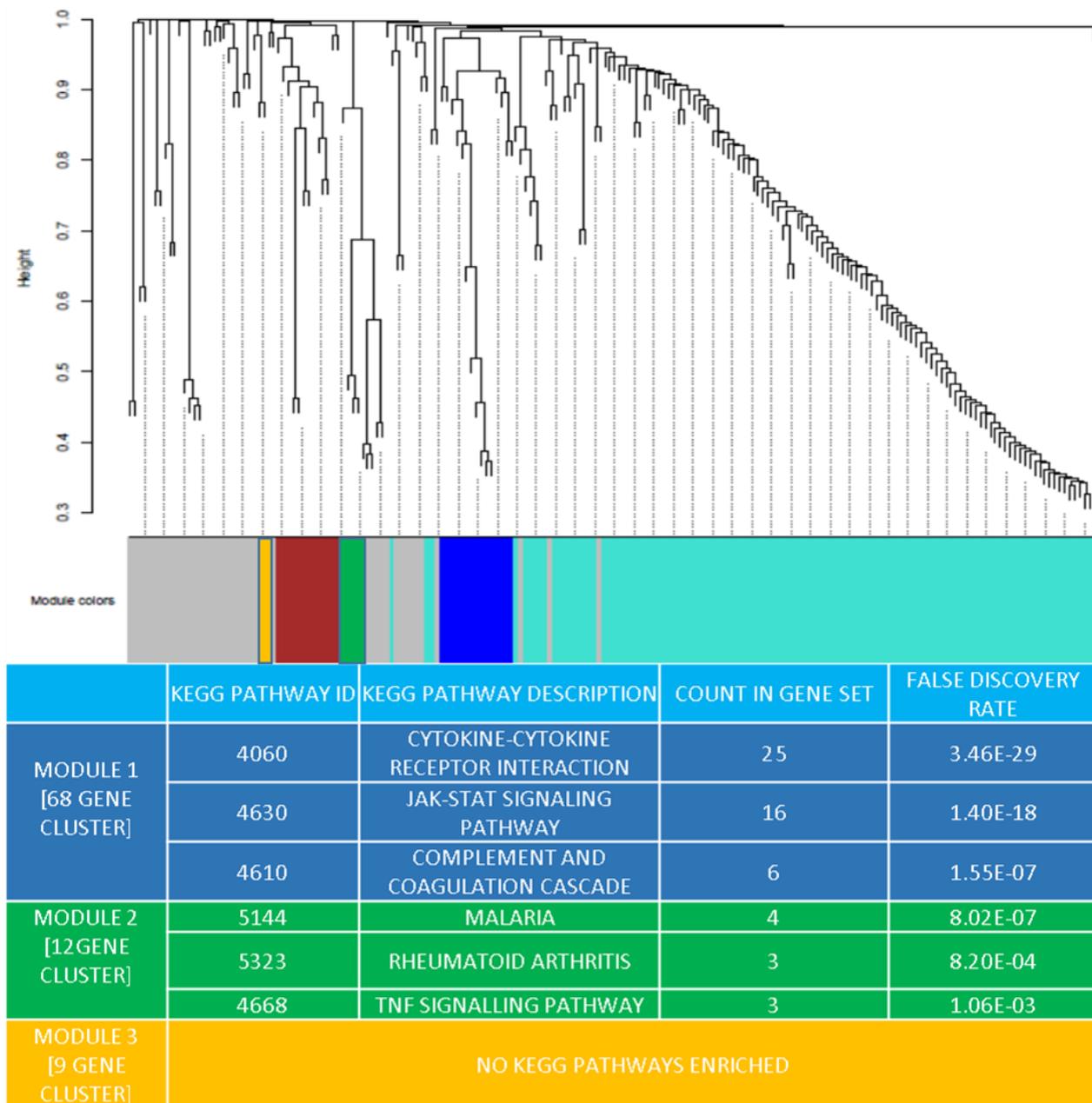


Figure 3. Network analysis dendrogram showing modules based on the coexpression topological overlap of genes related to inflammatory analytes and brain transcriptome. Color bars give information on module membership in they hold one or more analytes of significance identified in CSF and plasma. The table provides the related enrichment for KEGG pathways within these specific inflammation-related gene clusters of interest and false discovery rate.

tau, p-tau were different between ADNI and discovery cohorts (even though both met MCI-AD criteria) likely due to different analyte platforms for AD biomarkers. This likely impacts the correlation strengths between biomarkers and inflammatory analytes. However, these differences in absolute correlations did not notably impact the relative ordering/ranking of analyte correlations between the cohorts.

The relative ordering of the CSF inflammatory analyte correlations with CSF t-tau and p-tau was significantly high between the ADNI and discovery cohorts, with slightly lower correspondence for Aβ42 and plasma analytes. Furthermore, in the synergistic subgroup analysis after FDR correction, among the analytes with at least 50% of values above the limit of detection in the discovery panel, 30% were replicated in ADNI CSF. The

functional analysis of synergistic analytes in ADNI also independently pointed to shared inflammatory pathway activations as in the discovery cohort.

The differences in analyte correlation strengths between the discovery and the ADNI cohorts could be related to number of potential factors, (1) given the well-known challenges in standardization of AD biomarker quantification and these measurements being undertaken by necessity across different laboratories (Table 1) (2) Differences in patient recruitment characteristics: a memory clinic sample of MCI subjects with notable cognitive concerns in the discovery cohort, versus a longitudinal MCI cohort in ADNI with slightly lower CDR-SB and higher MMSE (Table 1). This could be related to an earlier stage of MCI-AD or potential biases against atypical forms of AD in ADNI (e.g., frontal variant, logopenic aphasia) c) Fewer shared correlation trends in the plasma than the CSF between the two cohorts (Fig. 2), also supports the possibility that these differences could be related to concomitant environmental exposures and/or medical comorbidities that were different between the cohorts, that could potentially have had a stronger effect on the plasma analyte levels than CSF analytes.

Brain transcriptome replication

Our use of AD brain transcriptome to evaluate the enrichment of inflammatory pathway activation provides additional level of confidence in the key inflammation-related pathways identified. The correspondence in key inflammatory analyte genes, and inflammatory pathways between brain, CSF, and plasma (A2M, SCF, MMP2, F7, FTH1, CRP, AAT, TNFRSF1B, B2M, TIMP1, ICAM1) following these results also provides a starting point for further experimental investigation of targeted inflammatory analytes in the CSF/ plasma knowing that a high degree of conserved biology exists with the brain.

Key analytes of note

Tumor necrosis factor receptor 2 (TNFR2)

Among the TNF signaling pathway analytes, TNFR2 had a consistently high correlation value to CSF t-tau, p-tau, and NSE. TNFR2 is expressed primarily in immune and endothelial cells. Signaling through TNFR2 activates inflammatory and prosurvival signaling pathways through subsequent activation of cellular inhibitor of apoptosis (cIAPs) and the NF- κ B pathway.³⁸ Interestingly, prior reports note a negative correlation between CSF levels of proinflammatory TNF α and t-tau.³⁹ Unlike TNFR2, it is notable that TNF α and related proinflammatory cytokines IL-6 and IL-1 β did not meet the limit

of detection in greater than 50% of subjects in both the cohorts used in the analysis. Taken together these suggest a cell protective role for the TNFR2 mediated pathway in MCI-AD. Additional analytes that correlated to the neurodegeneration markers are known to be part of the TNF signaling pathways mediating downstream functions including leukocyte recruitment across the blood brain barrier (CCL2), remodeling of extra cellular matrix (MMP3), cell adhesion (VCAM1), and vascular effects (VGEF).^{38,40}

Stem cell factor (SCF)

SCF levels also correlated highly to CSF t-tau and p-tau. It is a hematopoietic growth factor that is critically involved in regulation of blood cell production and mobilization of bone marrow stem cells and cell migration.⁴¹ SCF is highly overexpressed by neurons at sites of brain injury. It has been noted to mediate chemoattractant activity for neural stem/progenitor cell migration and thought to play a repair role in models of stroke.⁴² In the context of AD it has been reported to be low in the CSF and plasma of AD patients⁴³ and therapeutic effects of systemic SCF administration in mice models have shown promise in reducing amyloid.⁴⁴

The analytes related to the Complement and Coagulation pathways that were strongly related to CSF A β 42 include α -1-antitrypsin, plasminogen activator inhibitor (PAI) Type 1, and von Willebrand factor in the CSF and α -1-antitrypsin, complement factor 3 and coagulation factor VII in the plasma. The main consequences of the activation of this pathway are the opsonization of pathogens, the recruitment of inflammatory and immunocompetent cells, and the direct killing of pathogens.³⁸ The association between levels of these analytes and A β burden has been noted in multiple previous studies.^{45–48} Among the other inflammatory analytes of significance in our results, the relationships between AD diagnosis and CCL2,¹¹ MMP2,⁴⁹ and VGEF⁵⁰ have been previously reported, whereas VCAM1 has been reported in relation to both AD and vascular cognitive impairment.⁵¹

Even as TNF- α , IL-1 β , IL-6 and, IL-12 did not meet threshold for analysis, among proinflammatory cytokines that met analysis threshold, CSF and plasma CRP had negative correlations with CSF t-tau in both cohorts, whereas IL-18 in the plasma had negative correlations with CSF t-tau in both cohorts (Fig. 2). The direction of correlation in the above two analytes were consistent between the ADNI and discovery cohorts. In the plasma, the lack of some classical proinflammatory analytes as reported in previous AD meta-analysis, is notable.¹⁸ No prior studies have evaluated inflammatory analytes measured both in CSF and plasma in relation to CSF AD

biomarker levels, using Luminex technology to enable direct comparisons. Additionally as some of the prior studies lacked data on clinical variables that could impact proinflammation markers including comorbidities and concomitant medications (evaluated in this study), that could also account for difference in results.

Another inference from our CSF data is that, at the MCI stage of AD, TNF pathway cytokines that have been noted in AD animal and in vitro models in relation to amyloid,⁵² were here best correlated not to CSF A β 42 levels but to CSF t-tau, p-tau, and NSE pointing to potential TNF pathway activation in relation to beta amyloid deposition starting earlier in preclinical AD⁵³ that warrants investigation.

Strengths and limitations

There are several differences between the current study design and previous reports. First, even though some prior reports had a larger number of subjects than this study, they often lacked characterization by AD biomarkers, and often focused on the dementia stage of AD, both of which could potentially add confounders to the results. Furthermore, none of these previous studies had a validation cohort to evaluate replicability of results on the same analysis platform. Additionally the methods used in prior analyses were based on in-house methodologies, mostly ELISA, and not from a clinically validated shared resource that others could replicate their results against, limiting the generalizability. Second, there are concomitant CSF and plasma measurements to evaluate both peripheral and CNS-related inflammation changes in this report. Third, this study has multiple internal and external validity checks to account for quality of data and measurements. Fourth, we were able to go beyond single analyte associations to meaningfully assess multiple analytes and narrow our focus to key activated biological pathways. Fifth, we could assess the relevance of functional pathways identified in CSF and plasma across two different cohorts and for their activation in the AD brain tissue as well and validate these results.

Despite these strengths both Type I and II errors have to be considered in this study where we do not confirm some plasma analyte results reported previously, while other analytes (e.g., YKL-40) were not analyzed in the RBM MAP. Our results pass a stringent multiple comparisons cut-off but it is possible that with weaker enrichment patterns other analytes of significance may become more salient with increased sample sizes. Lack of neuropathologic confirmation of diagnosis also limits our understanding of the role for mixed pathology.

Conclusion

We report key inflammation-related pathway activations related to the TNF signaling pathway and complement and coagulation cascade were conserved in plasma, CSF and brain tissue in symptomatic AD. A cell protective rather than the proinflammatory analyte profile predominates in the CSF at the MCI-stage of AD in relation to neurodegeneration markers. Exploring their modulation toward therapeutic outcomes could be of clinical interest.

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Author Contributions

1. Research Project: A. Conception, B. Organization, C. Execution; 2. Statistical Analysis: A. Design, B. Execution,

C. Review and Critique; 3. Manuscript Preparation: A. Writing the First Draft, B. Review and Critique.

J.A.P.: 1) A, B, C 2) A, B 3) A

B.T.L.: 1) A 2) C 3) B

S.M.: 2) A, B 3) B

J.B.: 2) A, B 3) B

L.M.B.: 1) B, 2) C, 3) B

S.M.R.: 1) B, 2) C, 3) B

M.C.: 1) B, 2) C, 3) B

J.B.L.: 1) B, 2) C, 3) B

Conflict of Interest

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Supporting Information

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

Figure S1. Plot noting analytes that met lower limit of detection in at least 50% of subjects.

Figure S2. Cluster plot noting correlation coefficients of inflammatory analytes in plasma and CSF with AD biomarkers CSF t-tau, p-tau, and A β 42 in the discovery cohort.

Figure S3. Scatter plot of individual analyte correlations between ADNI and discovery cohort.

Table S1. Additional clinical and environmental characteristics of the discovery cohort.

Table S2. Summary statistics of analyte markers in plasma and CSF.

Table S3. Agreement statistics for correlations between inflammatory analytes and AD biomarkers between ADNI and discovery cohorts.

Table S4. Analyte subgroup analysis with most significant inflammatory analytes in the ADNI cohort.

Table S5. Significant KEGG pathways enriched in synergistic analyte analysis in discovery cohort.