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RESEARCH ARTICLE

Inflammatory pathway analytes predicting rapid cognitive decline in MCI stage of Alzheimer's disease

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Abstract

Objective: To determine the inflammatory analytes that predict clinical progression and evaluate their performance against biomarkers of neurodegeneration. **Methods**: A longitudinal study of MCI-AD patients in a Discovery cohort over 15 months, with replication in the Alzheimer's Disease Neuroimaging Initiative (ADNI) MCI cohort over 36 months. Fifty-three inflammatory analytes were measured in the CSF and plasma with a RBM multiplex analyte platform. Inflammatory analytes that predict clinical progression on Clinical Dementia Rating Scale-Sum of Boxes (CDR-SB) and Mini Mental State Exam scores were assessed in multivariate regression models. To provide context, key analyte results in ADNI were compared against biomarkers of neurodegeneration, hippocampal volume, and CSF neurofilament light (NfL), in receiver operating characteristic (ROC) analyses evaluating highest quartile of CDR-SB change over two years (≥3 points). **Results**: Cerebrospinal fluid inflammatory analytes in relation to cognitive decline were best described by gene ontology terms, natural killer cell chemotaxis, and endothelial cell apoptotic process and in plasma, extracellular matrix organization, blood coagulation, and fibrin clot formation described the analytes. CSF CCL2 was most robust in predicting rate of cognitive change and analytes that correlated to CCL2 suggest IL-10 pathway dysregulation. The ROC curves for ≥3 points change in CDR-SB over 2 years when comparing baseline hippocampal volume, CSF NfL, and CCL2 were not significantly different. **Interpretation**: Baseline levels of immune cell chemotactic cytokine CCL2 in the CSF and IL-10 pathway dysregulation impact longitudinal cognitive and functional decline in MCI-AD. CCL2's utility appears comparable to biomarkers of neurodegeneration in predicting rapid decline.

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Introduction

Alzheimer's disease (AD), which often presents early in its course with episodic memory loss, is the most common cause of dementia. It is increasingly recognized that there is considerable heterogeneity in AD phenotype and clinical trajectories.[1,2] Molecular factors that underpin this heterogeneity, however, remain ill-defined. Increasing evidence suggests that inflammatory pathways may regulate AD progression.[3–6] There has been a significant increase in interest for evaluating inflammatory changes in clinical AD with several studies reporting inflammation related changes in AD clinical cohorts over the last 5 years.[7–10]

There are also some key gaps in clarifying the role of inflammation across multiple clinical studies. These gaps include determining which specific peripheral and central immunological analytes and related pathways impact rate

of cognitive decline in human AD. There have been challenges in interpreting these results against longitudinal rate of disease progression. Often, the directionality and magnitude of these associations on clinical outcomes also often differ, likely due to the use of a small number of measured inflammatory analytes, varied methodologies, and different stages of disease.[6,11–16] In addition, it is unclear how any of these analytes compare with other widely used biomarkers of neurodegeneration, MRI hippocampal volume, and the novel biomarker neurofilament light (NfL) on disease progression.

To help identify inflammatory pathways or networks of inflammatory analytes pertinent to cognitive decline in AD, it is crucial to develop approaches that evaluate multiple analytes concomitantly and interrogate their clinical significance when expressed together. We therefore have taken a systematic approach to answer these gaps in mild cognitive impairment (MCI) with AD consistent CSF biomarkers, (MCI-AD) in whom the nature of inflammatory changes were characterized by the same multiplex panel of inflammatory analytes in the both the CSF and plasma. After evaluation in our Discovery cohort, we validated the results among MCI patients in the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort. Using bioinformatics and classical statistical tools, we determined (1) the key inflammatory analytes at baseline that best predict future cognitive decline, (2) the biological pathways most likely disrupted in relation to the above analytes and (3) how they compare to neurodegeneration biomarkers, MRI hippocampal volume, and cerebrospinal fluid (CSF) NfL. We tested the hypotheses that (1) a proinflammatory analyte profile at baseline would relate to a faster rate of longitudinal clinical progression in the MCI-AD and (2) key inflammatory analytes have at least comparable utility to neurodegeneration biomarkers in predicting future cognitive decline.

Materials and Methods

Discovery cohort

Forty-eight MCI-AD patients at baseline in whom the diagnosis of MCI-AD with CSF Aβ_{42} and p-tau$_{181}$ levels consistent with a diagnosis of AD and consensus evaluation of two neurologists and a neuropsychologist the details of which have been published previously.[17,18] The study was approved by the Cleveland Clinic Institutional Review Board. Eight patients did not complete the longitudinal evaluations due to nonmedical reasons by their personal choice. The ADmark® Alzheimer's evaluation uses sandwich Enzyme Linked Immunosorbant Assay(ELISA) kits [Innotest β-amyloid[1-42], Innotest hTAU-Ag, Innotest Phospho-Tau[181P], Innogenetics, Ghent, Belgium]. *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). Table 1 provides data on the Discovery cohort demographics. Inclusion, exclusion criteria are specified in Data S1. Additional clinical and environmental factors have been documented in Table S1 (Fig. 1).

Cognitive and functional measures

Mini–Mental State Examination (MMSE),[19] and Clinical Dementia Rating scale (CDR-SB)[20] were conducted to characterize the degree of their baseline cognitive and functional deficit. Mini–Mental State Examination and CDR-SB

Table 1. Demographics of Discovery and ADNI cohorts.

	Discovery (N = 48)		ADNI (N = 134)		
	N	Statistics	*N*	Statistics	*P*-value
Age at enrollment	48	68.1 ± 7.3	134	74.9 ± 7.2	***<0.001*** [a1]
Gender	48		134		0.23[c]
Male		28 (58.3)		91 (67.9)	
Female		20 (41.7)		43 (32.1)	
Years of education	48	16.0 [12.5, 18.0]	134	16.0 [14.0, 18.0]	0.27[b]
APOE ε4positive	48	37 (77.1)	134	72 (53.7)	***0.005***[c]
MMSE - baseline	48	24.8 ± 3.1	134	26.9 ± 1.8	***<0.001*** [a2]
CDR-SB - baseline	48	2.1 ± 1.2	134	1.5 ± 0.89	***0.002*** [a2]
CSF AB42, pg/mL	48	305.9 [216.1, 367.1]	134	144.5 [129.0, 171.0]	
CSF t-tau, pg/mL	48	454.3 [335.2, 771.3]	134	90.6 [67.8, 134.0]	
CSF p-tau, pg/mL	48	79.6 [59.3, 104.6]	134	35.7 [23.0, 45.8]	

Statistics presented as Mean ± SD, Median [P25, P75], *N* (column%).
P-values: a1 = *t*-test, a2 = Satterthwaite *t*-test, b = Wilcoxon Rank Sum test, c = Pearson's chi-square test, d = Fisher's Exact test.
P-value < 0.05 is noted in bold.
CDR-SB, Clinical dementia rating scale-sum of boxes; MMSE, Mini mental state exam.

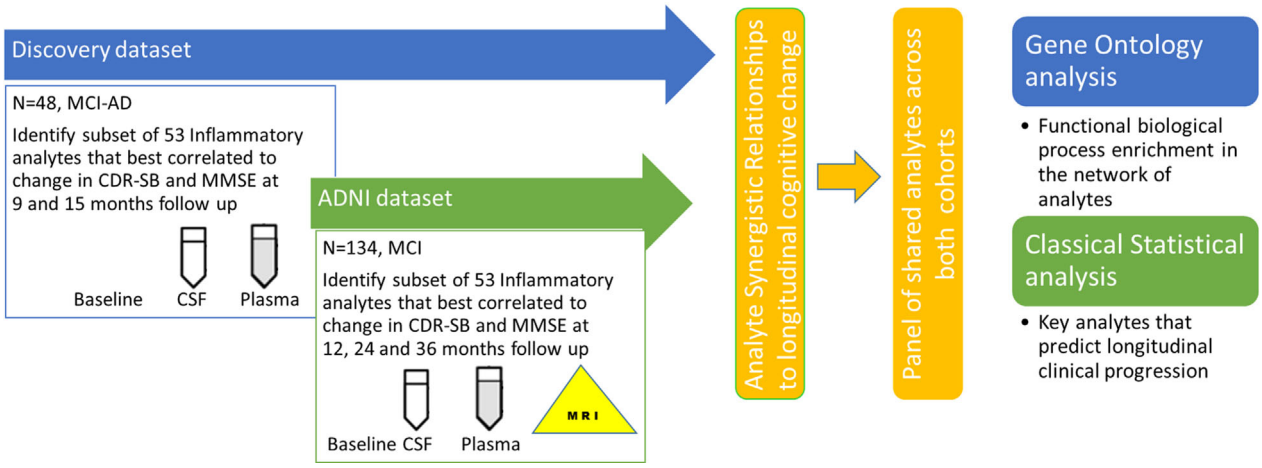

Figure 1. Methodological overview.

scores were also evaluated longitudinally to evaluate cognitive change at 9 and 15 months from baseline evaluations.

Inflammatory biomarkers

The biomarker analysis protocol used in this study has been previously described (18). In brief, 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. Cerebrospinal fluid and plasma samples were collected contemporaneously. Only those analytes with at least 50% response rate above the limit of detection in the Discovery cohort were included for further analysis.

ADNI validation cohort

Alzheimer's Disease Neuroimaging Initiative is a longitudinal multicenter study designed to develop clinical, imaging, genetic, and biochemical biomarkers for the early detection and tracking of AD. Alzheimer's Disease Neuroimaging Initiative 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/.

The demographics at baseline among the subset of all 134 ADNI MCI participants who had CSF and plasma multiplex data were used in the validation analysis are shown in Table 1. Longitudinal cognitive evaluations in the ADNI cohort documented at 12, 24, and 36 months were included in the analysis. AD CSF biomarker data were downloaded from http://adni.loni.usc.edu/ in which the CSF Aβ1-42, t-tau, and p-tau181 concentration data were generated using the Research Use Only (RUO) INNOBIA AlzBio3 immunoassay (Fujirebio, Belgium). Median values for each subject were used in the analysis. Amyloid-positivity based on a published, autopsy-confirmed cutoff value (<192 pg/mL) were used in a subgroup analysis to define MCI-AD[21,22] of which there were 106 participants (see Data S1). Cerebrospinal fluid 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. CSF NFL was measured using a sandwich ELISA method and provided as pg/ml (NF-light ELISA kit, UmanDiagnostics AB, Umeå, Sweden), as described previously. The lower limit of quantification for this assay was 50 ng/L.[23] Subject data quality was checked.

Bioinformatics and statistical analysis

Ontology analysis and network analysis to identify inflammatory analytes

Our final comprehensive list of 53 candidate inflammatory analytes (from 86 analytes in RBM MAP platform in the Discovery cohort) is provided in Table 2 and has been previously described[18] and rationale describing selection is in Data S1.

Subgroup searching for analyte synergistic relationships

Univariate analyses often fail in validation as they are trained on the specific patient dataset of the discovery

Table 2. List of inflammatory analytes analyzed in relation to longitudinal cognitive change.

RBM Name	Gene	RBM Name	Gene
1. Alpha-1-Antitrypsin (AAT)	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 (NSE)	ENO2
16. Interferon gamma	IFNG	42. Plasminogen Activator Inhibitor 1 (PAI-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

project, and average the heterogeneities present, while the validation cohort may have differences in the levels of individual analytes from multiple environmental factors. Network biology methods, specifically network based biomarker models, can effectively integrate heterogeneities at the patient level and provide robust validation across cohorts. 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 cognitive change measures.[18,24,25] 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 from among all analytes that met the 50% detection threshold and computing the correlation values. 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 to aggregate analyte levels than the actual response.

Functional pathway analysis on analytes of interest

The analytes of significance identified as being shared across both the Discovery and ADNI cohorts in at least one time point in the analyte subgroup search above were entered into STRING: functional protein association networks for pathway enrichment analysis.[26] The most significant GO terms related to biological process identified in STRING ($P < 0.05$) were summarized into nonredundant hierarchical terms by their semantic similarity via ReviGO,[27] using SimRel[28] as the clustering algorithm with a similarity measure of 0.70, with the whole Uniprot database providing GO term sizes and reported in appropriate plots. The Reactome pathway database was used in enrichment analysis to identify targeted analyte pathways of interest in any secondary analysis.[29]

Structural MRI acquisition and processing

ADNI 1.5-T MRI scans from MCI subjects performed at baseline were processed using cross-sectional FreeSurfer (version 5.1.0, default parameters).[30] The processing included conformation to isotropic cubic mm resolution, bias field correction, segmentation of the hippocampi

from which the bilateral hippocampal volume was computed, and estimation of total intracranial volume.[31] The baseline hippocampal volume was corrected for head size by division by the intra cranial volume.

Clustering analysis

To further provide biological context to the key shared analyte robustly identified across both ADNI and Discovery cohorts it was subjected to consensus clustering, an unsupervised class discovery approach to identify co-occurring analytes.[32] Using the quantitative values of the analytes, the number and possible memberships to clusters were found for each cluster. Cluster size for each group was fixed where relative increase in consensus was observed to have no appreciable increase. The analyte groups were tested for enrichment in pathways using ClusterProfiler tool using the Reactome Pathway Database[33] and the top five enriched Reactome pathways were interpreted.

Statistical analysis

Given the limited number of conversion events (dementia onset or highest quartile of CDR-SB change) during intermediate follow-ups (9 and 12 months) in both the Discovery and ADNI MCI cohorts, neither Cox proportional hazards model nor a time-dependent receiver operating characteristic (ROC) were found to be adequate for the data. With the Discovery sample size, and conservatively choosing a Bonferroni corrected significance level of 0.005 to allow for up to 10 key analytes to be compared, there would be 80% power to detect correlations between analytes and changes in cognitive measures that exceed 0.5. Key inflammatory analytes shared between both cohorts, curated from the synergistic subgroup analysis described previously and used for functional pathway analysis, were next evaluated in predicting cognitive change after adjusting for age, sex, baseline MMSE, and *APOE*ε4 status (present vs. absent). With five adjustment factors, the Discovery cohort models included no more than three biomarkers concurrently to maintain a 5 to 1 ratio of observations to variables as noted in prior statistical literature.[34] Two statistical approaches for variable selection were used. First, a best subset regression analysis was used to identify two to three biomarkers that best predicted outcome. In this approach, the third biomarker solution was used only if including the third biomarker increased the R^2 by at least 1%. As an alternative, penalized regression using LASSO regression was performed, using Akaike information criterion (AIC) as the stopping rule. In general, as the LASSO approach was very conservative the best subset results are presented.

A log (base 2) transformation allowed Pearson correlations to be fit for exploratory univariate analysis. Along with estimates of correlation, 95% confidence intervals and p-values with false discovery rate (FDR) adjustment were calculated. Normality of biomarkers was evaluated using Shapiro–Wilk tests and graphical methods. Sensitivity analyses were also performed to evaluate the robustness of the key analytes of significance. All analyses were performed at each time point using the patients with available cognitive change measures at that point. Separate analyses were performed at each time point because we hypothesized that the biomarkers related to cognitive change may change over time and the sample size did not allow for modeling of interactions to capture these complex relationships. We chose not to impute change measures for missing responses as doing so would not improve the information available.[35]

Classical ROC analyses were performed for subjects only in the ADNI cohort, to explore if key CSF inflammatory analytes were comparable to hippocampal volume and CSF NfL in their clinical utility as all three data were available. Analyses were performed for a base model with above three variables alone and for an adjusted model that included age, sex, education years, APOEε4 status, and CSF Aβ/ptau ratio. The area under the ROC curve (AUC) was maximized in these analyses. In this setting, the AUC measures the intrinsic ability of the analytes to discriminate between subjects who progress ≥ 3 CDR-SB points, the highest quartile CDR-SB change in the ADNI cohorts for two year follow-up. Analysis was performed using SAS software (version 9.4), R software (version 3.x; Vienna, Austria), and SPSS (Version 22.0. Armonk, NY: IBM Corp) an overall significance level of 0.05 was assumed.

Results

Subject demographics of the Discovery and ADNI cohorts are presented in Table 1.

Analyte subgroup analysis for synergistic relationships in discovery and ADNI cohorts

Analyte levels that show higher correlation when considered together rather than the individual component analytes (synergistic analyte analysis) are reported in Tables S3 and S4 and Figures 2A and 3A. The Discovery cohort had a similar number of analytes compared with the ADNI cohort that relate to cognitive changes in the CSF and in plasma. The analytes noted in relation to CDR-SB change in a consistent direction among both cohorts in the CSF included CCL2, CCL4, and FGA, while in plasma the shared analytes in a consistent

direction included APOA1, BDNF, and vWF. Among both cohorts, the CSF analytes noted in relation to MMSE change in a consistent direction across both cohorts included AAT, MMP3, and CRP, while in plasma MMP2, CXCL8, A2M, and Factor VII were best correlated to MMSE change consistently across both cohorts (Figs. 2A and 3A).

Functional pathway analysis

The analytes that significantly correlated to at least one time point of longitudinal follow-up in both Discovery and ADNI cohorts in a consistent direction within the subgroup synergistic analysis were ranked as the most robust for functional pathway analysis. These analytes (for CSF: AAT, CCl2, CCL4, CRP, FGA, and MMP3 and for plasma: A2M, APOA1, BDNF, CXCL8, F7 MMP2, and vWF) were entered into STRING for functional pathway enrichment analysis for CSF and plasma in different runs. In the CSF, the largest clusters were most representative of natural killer cell chemotaxis, and regulation of endothelial cell apoptotic process. While in the plasma, clusters were representative of extracellular matrix organization, blood coagulation and fibrin clot formation, and platelet degranulation (Figs. 2B and 3B).

Statistical analysis

Univariable analysis in CSF and plasma

In a complementary analysis, univariable Pearson correlations after FDR correction in the Discovery cohort, only CSF levels of CCL2 positively correlated to change in CDR-SB scores at 15 months, and after adjusting for covariates (age, sex, baseline cognitive score, *APOE ε4* status), the association of CCL2 was still significant (Table S4A).

Within the ADNI cohort, there were no significant CSF analytes on univariable analysis that related to change in CDR-SB or MMSE scores after adjusting for covariates. In the plasma, only levels of MMP2 was negatively correlated to change in MMSE scores at 24 months, and the association was still significant after adjusting for covariates (age, sex, baseline cognitive score, *APOE ε4* status) while applying the FDR correction (Table S5).

Multivariable models CSF and plasma

Across both cohorts, CSF CCL2 was the most robustly selected parameter being chosen in 9 out of 10 models, and was significant at the 0.05 level in five models. MMP3 was chosen in four models and met significance threshold

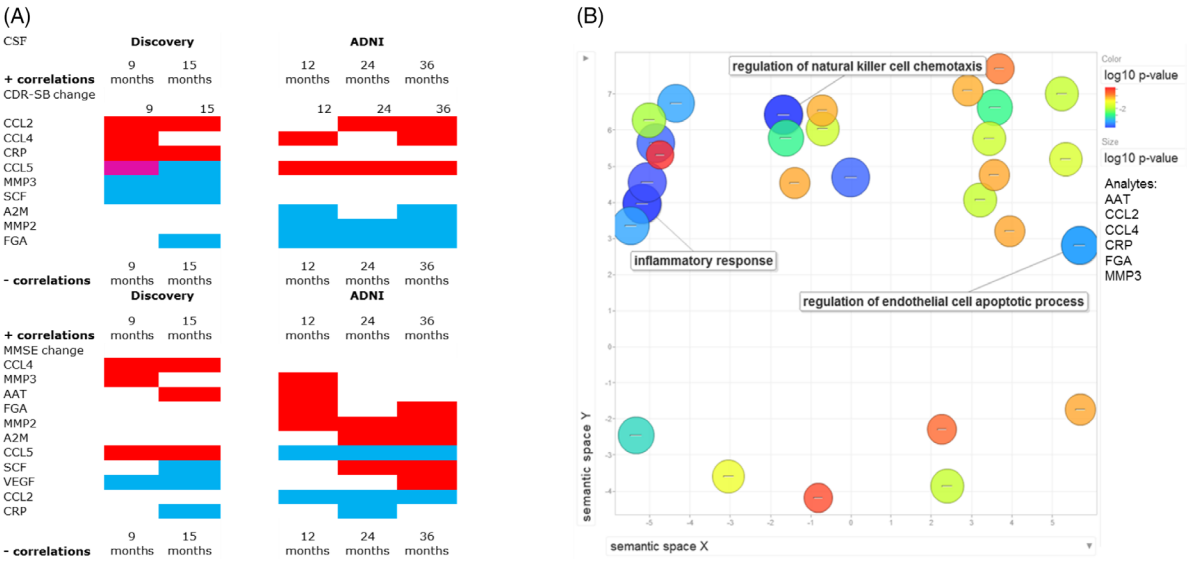

Figure 2. (A) Heat map denoting significant analytes from ADNI and Discovery datasets in the CSF from the subgroup synergistic analysis correlated to cognitive change (CDR-SB or MMSE) for 9,12,15,24, and 36 months. Red: positive correlation, Blue: negative correlation, Magenta: has representation in both positive and negative correlation network of analytes. Given that higher CDR-SB is worse cognition and function while lower MMSE is worse cognition, a positive correlation in CDR-SB relates to worsening cognition while the negative MMSE correlations relates to worsening cognition. (B) Abundance of Gene Ontology (GO) terms related to biological processes that enrich for key shared analytes between ADNI and Discovery datasets in the CSF in relation to cognitive change (CDR-SB or MMSE). Analytes included: AAT, CCl2, CCL4, CRP, FGA, and MMP3. GO term most representative of each cluster is noted.

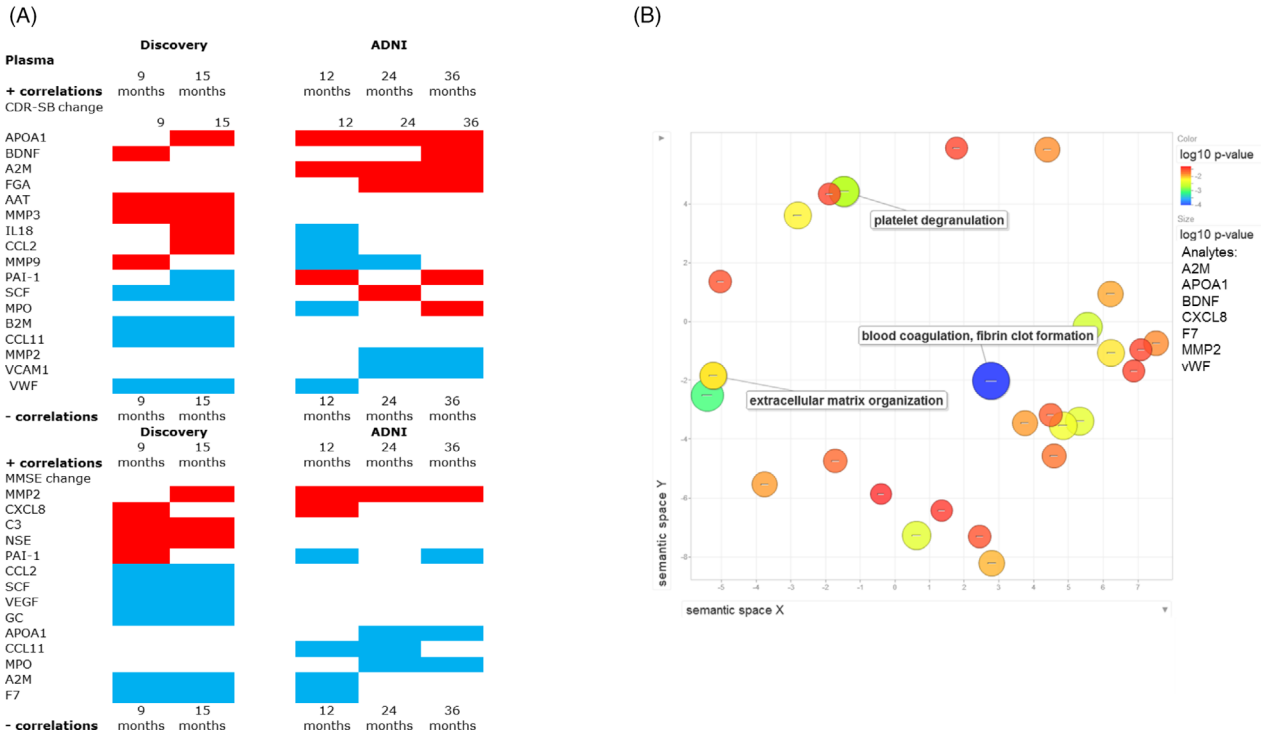

Figure 3. (A) Heat map denoting significant analytes from ADNI and Discovery datasets in the p*lasma* from the subgroup synergistic analysis correlated to cognitive change (CDR-SB or MMSE) for 9,12,15,24, and 36 months. Red: positive correlation, Blue: negative correlation, Magenta: has representation in both positive and negative correlation network of analytes. **3B:** Abundance of Gene Ontology (GO) terms related to biological processes that enrich for key shared analytes between ADNI and Discovery datasets in the plasma in relation to cognitive change. Analytes chosen for their consistency of response included: A2M, APOA1, BDNF, CXCL8, F7, MMP2, and vWF. GO term most representative of each cluster is noted.

in three models. CSF CCL4 selected in four models, was significant in only two models. In the Discovery cohort, the multivariable models explained 27–42% of the variability in cognitive change, while in ADNI, only 13–19% of the variability in cognitive change was explained with these models (Table 3). Additional sensitivity analysis to evaluate the role of extreme measures using a rank based analysis noted CSF CCL2 as significant in all prior models, MMP3 was no longer significantly related to CDR-SB change at 15 months.

In the plasma, a clear pattern replicating the findings between Discovery and ADNI data does not appear to be present in the multivariable models (Table 4). Across both cohorts, there were no analytes with correlations in opposing directions to the same cognitive change scores when correlations exceeded +/- 0.2 in the univariable models in CSF and plasma. In the multivariable models with MMSE, plasma BDNF and MMP2 differ in correlation direction between cohorts. Plasma MMP2 changes correlation direction within the ADNI cohort over time, indicating the instability of this effect when cognitive change is characterized by MMSE.

Inflammatory diseases and NSAID intake

No difference was noted in the analytes of significance when adjusted for current NSAID intake or when considering inflammatory diseases in clinical history within the Discovery cohort. Adjusting for individual CSF/plasma albumin ratio again noted no impact on key analytes of significance (analyses not presented).

Comparing inflammatory analytes in ADNI versus the Discovery cohort

The inflammatory analyte correlations to cognitive change within the Discovery cohort were larger than in the ADNI dataset (Tables S4 and S5). The analytes that were identified as correlating to at least one longitudinal cognitive measure across both datasets could be further validated after accounting for multiple covariates in the multivariable models (Tables 3 and 4). CSF CCL2 was identified as a key analyte shared between cohorts in relation to CDR-SB change. In a subgroup analyses of ADNI MCI participants with CSF Aβ42 < 192 pg/mL (and additionally CSF t-tau/

Table 3. CSF biomarkers of CDR-SB and MMSE change are shown for both cohorts at specified time points, after adjustment for age, sex, APOE status and education.

	CDR-SB change			MMSE change		
Time	Factor	Estimate (95% CI)	*P*-value	Factor	Estimate (95% CI)	*P*-value
Discovery cohort						
9 months	CCL2	1.23 (0.24, 2.22)	***0.016***	AAT	1.50 (−0.85, 3.85)	0.20
	MMP3	−0.97 (−1.64, −0.29)	***0.006***	CCL2	−1.54 (−4.09, 1.02)	0.23
				MMP3	2.05 (0.32, 3.78)	***0.022***
15 months	FGA	−0.27 (−0.73, 0.20)	0.25	AAT	1.53 (−1.24, 4.30)	0.27
	CCL2	2.82 (1.30, 4.34)	***<0.001***	CCL2	−2.36 (−5.47, 0.76)	0.13
	MMP3	−0.94 (−1.82, −0.05)	***0.039***	CCL4	1.48 (−0.15, 3.12)	0.073
ADNI Cohort						
12 months	AAT	0.37 (−0.10, 0.85)	0.12	CCL2	−1.45 (−2.54, −0.36)	***0.009***
	CCL4	0.30 (−0.03, 0.63)	0.071	FGA	0.49 (0.13, 0.86)	***0.008***
	FGA	−0.23 (−0.40, −0.05)	***0.011***			
24 months	CCL2	0.86 (−0.04, 1.75)	0.062	CCL2	−1.46 (−3.19, 0.26)	0.096
	CCL5	0.41 (0.02, 0.79)	***0.041***	CCL5	−0.43 (−1.18, 0.31)	0.25
				CRP	−0.26 (−0.65, 0.13)	0.20
36 Months	CCL2	1.43 (0.13, 2.73)	***0.031***	CCL2	−2.87 (−5.16, −0.58)	***0.015***
	CCL4	0.76 (0.01, 1.52)	***0.047***	CCL4	−1.36 (−2.71, −0.00)	***0.050***
	MMP3	−0.53 (−1.25, 0.19)	0.15	CCL5	0.74 (−0.50, 1.98)	0.24

P-value < 0.05 is noted in bold

Table 4. Plasma biomarkers of CDR-SB and MMSE change are shown for both cohorts at specified time points, after adjustment for age, sex, APOE status and education.

	CDR-SB Change			MMSE Change		
Time	Factor	Estimate (95% CI)	*P*-value	Factor	Estimate (95% CI)	*P*-value
Discovery cohort						
9 months	CXCL8	0.37 (−0.39, 1.12)	0.33	A2M	−4.24 (−7.67, −0.81)	***0.017***
	F7	−1.19 (−2.04, −0.34)	***0.007***	F7	−1.97 (−3.81, −0.13)	***0.037***
	MMP2	−0.70 (−1.80, 0.41)	0.21	BDNF	0.28 (−0.23, 0.79)	0.27
15 months	CXCL8	1.75 (0.66, 2.84)	***0.003***	A2M	−4.37 (−8.62, −0.12)	***0.044***
	APOA1	−1.41 (−3.75, 0.93)	0.23	F7	−1.83 (−4.26, 0.60)	0.13
	F7	−0.85 (−2.14, 0.44)	0.19	MMP2	−1.28(−4.47, 1.92)	0.42
ADNI Cohort						
12 months	A2M	0.66 (0.06, 1.27)	***0.032***	CXCL8	1.03 (0.34, 1.71)	***0.004***
	APOA1	0.28 (−0.13, 0.69)	0.18	APOA1	−0.61 (−1.50, 0.28)	0.18
				BDNF	−0.24 (−0.63, 0.15)	0.23
24 months	APOA1	0.57 (−0.08, 1.23)	0.086	APOA1	−1.16 (−2.35, 0.03)	0.057
	BDNF	0.23 (−0.05, 0.51)	0.10	MMP2	1.48 (0.88, 2.08)	***< 0.001***
	MMP2	−0.56 (−0.89, −0.22)	***0.001***			
36 months	A2M	1.73 (−0.28, 3.74)	0.090	A2M	−1.29 (−3.91, 1.33)	0.33
	MMP2	1.12 (−0.19, 2.42)	0.093	APOA1	−1.26 (−3.13, 0.61)	0.18

P-value < 0.05 is noted in bold.

Aβ42> 0.4), the direction of correlation between CCL2 and CDR-SB change on univariable analysis was consistent between 24 and 36 months and met significance threshold at 36 months (Table S6). From the multivariable analysis, a doubling of baseline CSF CCL2 levels in the Discovery cohort predicted a 2.8 point estimated increase in CDR-SB change at 15 months. A similar doubling in the ADNI MCI cohort predicted a 0.9 point increase of CDR-SB over 24 months and 1.4 point over 36 months, after accounting for age, sex, education, *APOE* ε4 status, and baseline cognitive scores (Table 3, Fig. 4).

To provide a biological context to the role for CCL2, we next explored the clustering of CCL2 to other inflammatory analytes. We note that in the Discovery cohort

CCL2 closely clusters with B2M, CXCL8, FGA, MMP2, TIMP1, and VCAM1 in the CSF (Fig. S1) and plasma CCL2 with BDNF, CCL4, CCL11, IL-18, PAI-1, and VEGFA (Fig. S2). These analytes in the CSF and plasma taken together are enriched in interleukin-10 (IL-10), interleukin-4 (IL-4), and interleukin-13 (IL-13) signaling pathways within the Reactome database (Tables S7 and S8). In the ADNI cohort, CSF CCL2 did not significantly cluster with other CSF analytes, while in the plasma, CCL2 and correlated inflammatory plasma analytes were enriched for the IL-10 signaling pathway (Fig. S3, Tables S9). Summary statistics of these analytes are provided in Data S1.

CSF versus plasma inflammatory analytes in predicting future cognitive decline

Receiver operating characteristics based on the logistic regression models determined the utility of CCL2 compared with baseline CSF NfL and hippocampal volume to predict the highest quartile of CDR-SB change over two years in the ADNI cohort. The area under the curve (AUC) of the adjusted model (age, sex, years of education, APOE $\varepsilon 4$ genotype, and CSF $A\beta_{1-42}$/p-tau) when it included CSF CCL2 was 0.66, CSF NfL was 0.63, and hippocampal volume was 0.69 (Fig. 5). The resulting AUCs and overlapping 95% confidence intervals were statistically not different for these biomarkers (Table S10). A nonsignificant correlation between CSF CCL2 and baseline hippocampal volume, $r = -0.15$, $P = 0.12$ was noted, while the correlation between CSF CCL2 and CSF NfL was $r = 0.19$, $P = 0.042$.

Discussion

This study undertook an unbiased approach to evaluate the role for inflammatory analytes on clinical progression using a multi-analyte panel with well characterized MCI patient cohorts and positive AD CSF biomarkers. Our results across two cohorts of baseline CSF CCL2 predicting rapid clinical decline lends credence to prior reports of CCL2 impacting clinical progression in AD.[13,36] In addition as a novel finding, by carefully characterizing chemokines that cluster closely with CCL2 in the CSF and plasma using bioinformatics tools, we are able to posit the relevance of IL-10 inflammatory pathway dysregulation as a correlate of clinical progression in MCI. This result is consistent with our initial hypothesis that a proinflammatory analyte profile at baseline would relate to a faster rate of longitudinal clinical progression in the MCI stage. Furthermore providing clinical context to these results, we note that baseline measurements of the cytokine CSF CCL2 has comparable discriminatory power to neurodegeneration markers, CSF NfL and MRI hippocampal volume, in predicting the highest quartile of CDR-SB change over two years (≥3 CDR-SB) in MCI-AD, but has limited correlation with either. Of note, 3 point CDR-SB change is over twice the change among

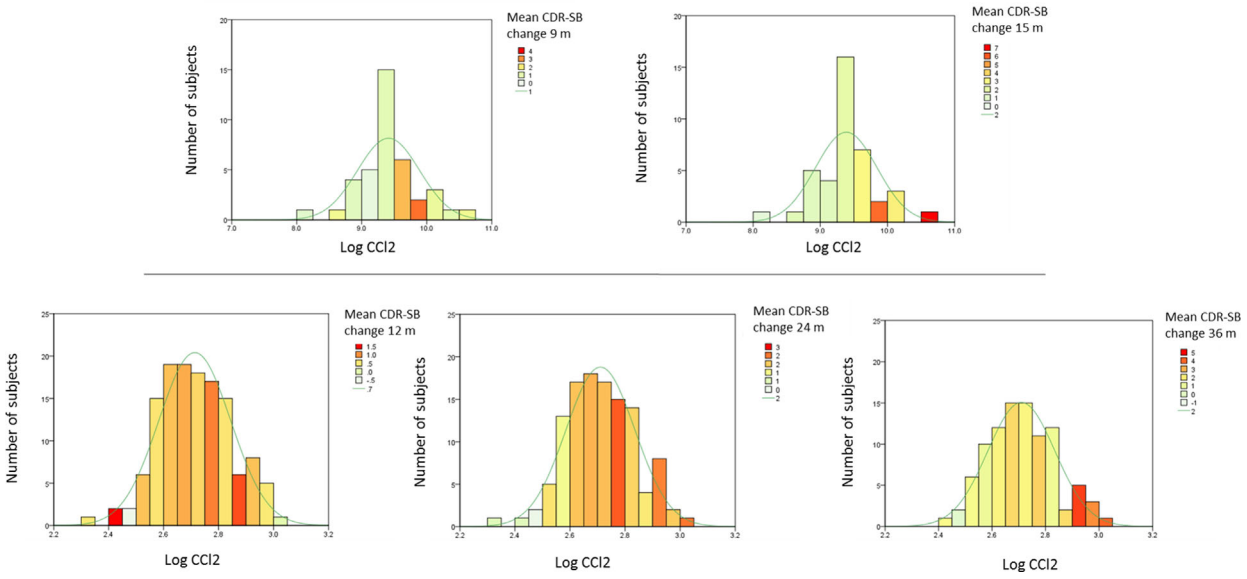

Figure 4. Histogram with normal distribution score of baseline CSF CCL2 levels, in relation to longitudinal CDR-SB change (in heat colors) and average CDR-SB change of cohort at each time point (as a line in legend). Data from Discovery cohort (9 and 15 months), ADNI (12, 24 and 36 months).

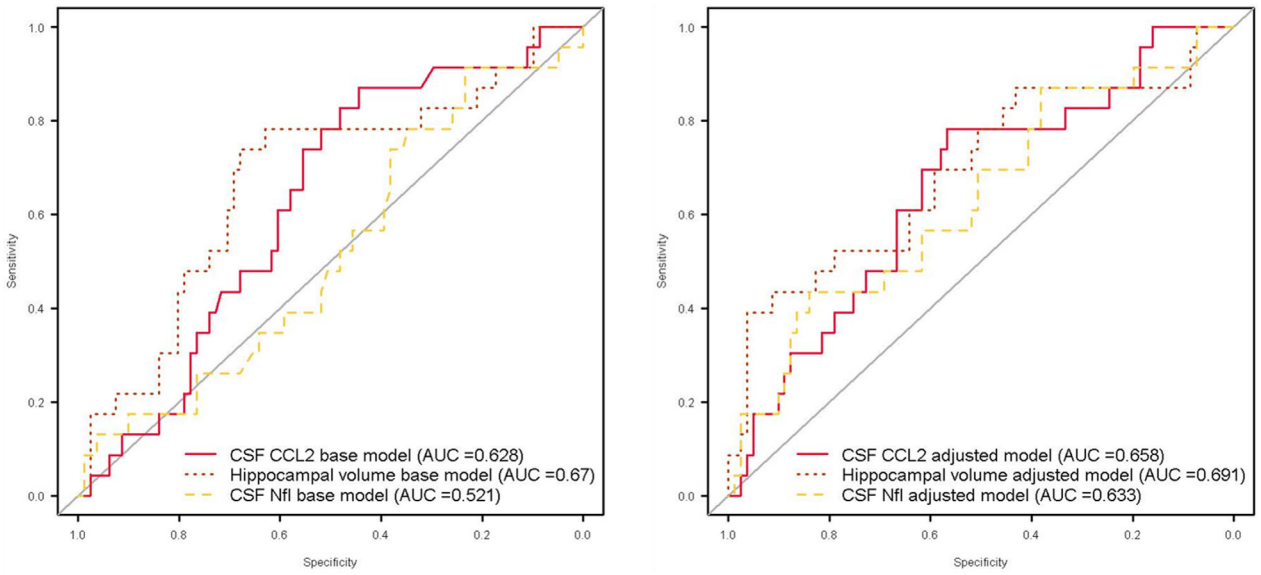

Figure 5. Receiver operating characteristic analysis curves denoting CSF CCL2, CSF NfL, and hippocampal volume for rapid cognitive decline (≥ 3 CDR SB change over two years, highest quartile among subjects) from ADNI cohort. Base models of the three biomarkers alone and adjusted models accounting for age, sex, years of education, APOE ε4 genotype, and CSF Aβ1–42/p-tau.

neuropathology confirmed AD patients in the National Alzheimer's Coordinating Center (NACC) database (Avg 0.9, SD 0.7, per year), when they met criteria for MCI at initial visit.[2]

CCL2 (also known as monocyte chemoattractant protein-1, MCP-1) is among the key cytokines that recruit monocytes to a site of inflammation. Infiltration of these blood derived immune cells toward Aβ plaque have been studied.[37,38] An increase in CSF CCL2 levels has been linked to the transition from MCI to AD and a faster rate of cognitive decline.[13,36] Plasma CCL2 levels have been observed to increase with the increasing severity of AD dementia and associated with a 2-year rate of cognitive decline in AD and MCI.[36,39] Among asymptomatic normal aging individuals, longitudinal increases in plasma CCL2 levels were associated with decline in memory[40] and associated with increased long-term risk of stroke in a meta-analysis of population studies.[41]

IL-10 Reactome pathway was enriched among the CSF and plasma inflammatory analytes that CCL2 clustered with in both ADNI and the Discovery cohorts, while IL-13 and IL-4 pathways were enriched only in the Discovery cohort. IL-10, IL-13, and IL-4 pathways are all associated with anti-inflammatory changes noted in relation to inhibition of autoimmunity and infections.[42,43] The downstream proinflammatory factors in the IL-10 Reactome pathway often suppressed by IL-10 were elevated. Given the Lower Limit of Quantification (LLOQ) of the CSF IL-10 assay only 3 of 48 patients in the Discovery cohort had measurable IL-10 levels, limiting the assay's utility in estimating IL-10's direct relevance in this context. In contrast to CCL2, the levels of MMPs were less consistently correlated to cognitive change in both cohorts.

CCL2 compared to neurodegeneration markers

To evaluate the utility of CSF CCL2 in predicting rapid cognitive decline, we compared its effectiveness against baseline CSF NfL and MRI hippocampal volume as they both provide complementary information. Imaging measures like hippocampal volume represent the magnitude of the neuropathologic damage accumulated over time, unlike CSF markers like NfL that reflect its production/clearance at one time point.[44] CSF NfL has been noted to correlate with rapid cognitive decline in MCI stage of AD and is thought to be related to degeneration of large-caliber axons.[45] MRI measure of hippocampal volume is accepted as an indicator of neurodegeneration in the A/T/N classification.[44] In the ROC analysis, CSF CCL2 had nonsignificant differences from CSF NfL and hippocampal volumes in predicting rapid cognitive decline in the adjusted models that included covariates of clinical importance. In the base model when these biomarkers were considered without adjustment for covariates, CSF CCL2 performed slightly better than CSF NfL (0.63 vs. 0.52), but was still within the 95% CI. The lack of correlation between CCL2 and neurodegeneration biomarkers suggests that they likely capture different pathobiological signatures contributing to cognitive decline in MCI-AD.

Inflammatory changes in the context of neurodegeneration markers and clinical progression

In our prior analysis, we had found TNFR2, SCF, and Ferritin strongly correlated to neurodegeneration markers and were expressed in the brain transcriptome.[18] In the current analysis, the above analytes were not significant in predicting longitudinal cognitive change within three years. The key inflammatory pathways are also likely to be distinct in different stages of clinical AD and needs further elucidation.

Even as migration of neutrophils toward amyloid plaques are noted in some mouse models of AD,[46] this is an area that needs future investigation in MCI-AD patients to determine if CCL2 determined immune cell migration plays a role in impacting cognitive outcomes. The study data also point to a wide variance in the inflammatory analyte levels within patients at the same stage of MCI-AD suggesting different degrees of inflammatory pathway dysregulation. Taken together these data suggests a promise for targeted therapies against key inflammatory pathways among patients in whom it is most dysregulated to have a significant clinical outcome within the time widows described in this study. The shared AUCs across CSF CCL2 and neurodegeneration biomarkers in predicting rapid cognitive change over two years but a lack of significant correlation between them, suggest they could play parallel roles in predicting disease progression.

Differences between Discovery and ADNI cohorts

The current study noted key shared inflammatory analyte correlations that were consistent between the Discovery and ADNI cohorts. The Discovery cohort analytes had larger correlation coefficients than ADNI in relation to degree of cognitive decline. The correlations of cognitive/functional change to CSF CCL2 are robust in the Discovery data at 15 months, and get stronger over the 3-year time of follow-up in the ADNI data. This may reflect a slower progressing population in ADNI, or an earlier stage of MCI in ADNI compared to Discovery cohort as noted in Figure 4 and Table 1. The common analytes meeting significance threshold in both ADNI and Discovery cohort in the CSF was CCL2 and in the plasma was CXCL8 (IL-8). CXCL8 was also the only analyte that clustered with CCL2 in both the Discovery and ADNI cohort plasma pathway analysis (Table S11). Additional differences between the two cohorts include differences in duration of follow-up, AD biomarker levels at baseline and 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 likely different medical and environmental biases, as noted in the less robust correspondence in plasma analytes compared to CSF between the two cohorts. Despite these differences in data collection and patient variables, key shared analytes were still identified at baseline to have a longitudinally clinical impact across both cohorts.

Strengths and limitations

The study's strengths include a) concomitant measurement of the same analytes in the CSF and plasma; b) well-characterized patients including clinical variables (inflammation, vascular risk factors and medications), *APOE* ε4 status, and AD biomarkers, longitudinal assessment of cognition and validation across two different cohorts with potentially different recruitment biases; c) multiple internal and external validity checks to account for quality of data and measurements, and d) going beyond single analyte associations to meaningfully assess multiple analytes and narrow our focus to key activated biological processes related to inflammation in AD with high confidence.

Despite these strengths, this study is not comprehensive in determining the profile of inflammatory analytes as some (e.g., YKL-40, sTREM2) were not analyzed. The Discovery cohort and ADNI longitudinal cognitive measurements do not have the same follow-up duration. Additional inflammatory pathways could also be contributing to cognitive outcomes than those posited following our analysis. We also have limited insight based on baseline measures alone, as they themselves could be dynamic and change longitudinally, contributing to variation in the temporal window of strongest cognitive outcome in different stages of AD in both cohorts.

Future studies are needed to evaluate potential inflammatory analytes/pathways not covered in this analysis. Our results pass a stringent multiple comparisons cutoff, 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 in MCI-AD.

Conclusion

We found that cognitive decline in MCI-AD was best predicted by CSF CCL2 and likely related to IL-10 pathway dysregulation in the CSF and plasma. Baseline CSF CCL2 has comparable utility to CSF NfL and hippocampal volume in predicting rapid cognitive decline. Exploring the triggers of this inflammatory response related to chemotaxis of immune cells and prospect of their modulation

provides a potential therapeutic opportunity that is of clinical interest in MCI-AD.

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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
BTL: 1) A 2) C 3) B
JB: 2) A, B 3) B
KL: 2) B
GB: 2) A, B 3) B
AP: 2) C, 3) B
LS: 2) C, 3) B
MN: 2) C, 3) B
LMB: 1) B, 2) C, 3) B
SMR: 1) B, 2) C, 3) B
MC: 1) B, 2) C, 3) B
JBL: 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. Discovery cohort CSF levels were clustered to reveal correlated measurements using Consensus clustering.

Figure S2. Discovery cohort plasma levels were clustered to reveal correlated measurements using consensus clustering.

Figure S3. ADNI cohort plasma levels were clustered to reveal correlated measurements using consensus clustering.

Table S1. Additional clinical characteristics of the Discovery and ADNI cohorts and environmental characteristics of the Discovery cohort.

Table S2. Analyte subgroup analysis with most significant inflammatory analytes in the Discovery cohort.

Table S3. Analyte subgroup analysis with most significant inflammatory analytes in the ADNI cohort.

Table S4. Univariate correlations between inflammatory analyte and cognitive change in the Discovery cohort after controlling for baseline cognitive score, age, sex, and APOE ε4 status.

Table S5. Univariate correlations between inflammatory analyte and cognitive change in the ADNI cohort after controlling for baseline cognitive score, age, sex, and APOE ε4 status.

Table S6. CSF CCL2 correlation to ADNI CDR-SB change after adjustment for baseline CDRSB score, age, sex, and APOE ε4 status with different AD biomarker thresholds.

Table S7. REACTOME pathways (Top 5) that enrich for CSF levels of CCL2 and its highly clustered analytes (B2M, CXCL8, FGA, MMP2, TIMP1, and VCAM1) in Discovery Cohort.

Table S8. REACTOME pathways (Top 5) that enrich for Plasma levels of CCL2 and its highly clustered analytes (AAT, BDNF, CCL4, CCL11, IL-18, and VEGFA) in Discovery Cohort.

Table S9. REACTOME pathways (Top 5) that enrich for Plasma levels of CCL2 and its highly clustered analytes AAT, BDNF CCL2, CCL4, CCL5,CXCL8,KITLG (SCF), and MPO in ADNI Cohort.

Table S10. ROC analysis looks at how CDR-SB progression ≥3 points over 2 years are predicted by CSF CCL2, CSF NfL, and hippocampal volume corrected for intracranial volume. Data from 88 patients in the ADNI dataset that had concomitant CSF CCl2, NfL, and hippocampal volume data at two years follow-up.

Table S10. Comparison of significant analytes in the pathway analysis and multivariable models.

Data S1. Supplementary methods and summary statistics.

