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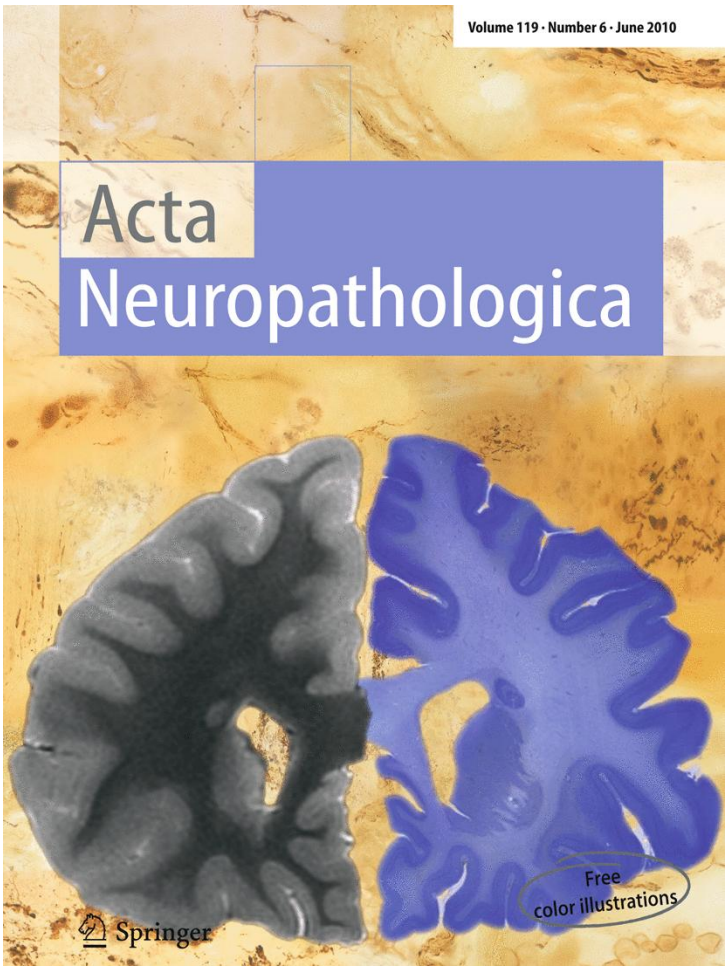

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ORIGINAL PAPER

Novel CSF biomarkers for Alzheimer's disease and mild cognitive impairment

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Abstract Altered levels of cerebrospinal fluid (CSF) peptides related to Alzheimer's disease (AD) are associated with pathologic AD diagnosis, although cognitively normal subjects can also have abnormal levels of these AD biomarkers. To identify novel CSF biomarkers that distinguish pathologically confirmed AD from cognitively normal subjects and patients with other neurodegenerative disorders, we collected antemortem CSF samples from 66 AD patients and 25 patients with other neurodegenerative dementias followed longitudinally to neuropathologic confirmation, plus CSF from 33 cognitively normal subjects. We measured levels of 151 novel analytes via a targeted multiplex panel enriched in cytokines, chemokines and growth factors, as well as established AD CSF biomarkers (levels of Aβ42, tau and p-tau$_{181}$). Two categories of biomarkers were identified: (1) analytes that specifically distinguished AD (especially CSF Aβ42 levels) from cognitively normal subjects and other disorders; and (2) analytes altered in multiple diseases (NrCAM, PDGF, C3, IL-1α), but not in cognitively normal subjects. A multiprong analytical approach showed AD patients were best distinguished from non-AD cases (including cognitively normal subjects and patients with other neurodegenerative disorders) by a combination of traditional AD biomarkers and novel multiplex biomarkers. Six novel biomarkers (C3, CgA, IL-1α, I-309, NrCAM and VEGF) were correlated

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with the severity of cognitive impairment at CSF collection, and altered levels of IL-1α and TECK associated with subsequent cognitive decline in 38 longitudinally followed subjects with mild cognitive impairment. In summary, our targeted proteomic screen revealed novel CSF biomarkers that can improve the distinction between AD and non-AD cases by established biomarkers alone.

Keywords Amyloid beta · Abeta42 · Diagnosis · IL-1α · MCI · NrCAM · PDGF · Resistin · TECK · TDP-43 · Tau

Introduction

Alzheimer's disease (AD), frontotemporal lobar degenerations (FTLD) and dementia with Lewy bodies (DLB) are major neurodegenerative disorders pathologically characterized by lesions composed of disease-specific misfolded proteins. Their clinical syndromes often have overlapping features, making antemortem prediction of pathology challenging. Yet, as specific disease-modifying therapies become available, it is increasingly important that such diagnoses be made. Analytes in cerebrospinal fluid (CSF) associated with AD pathology, such as total tau, tau phosphorylated at threonine 181 (p-tau$_{181}$) and Aβ1-42 (or Aβ42), offer the potential for more accurate diagnosis, although cognitively normal elderly subjects could have altered levels of these traditional AD biomarkers [20, 24, 26]. Peptides in common inflammatory and apoptotic pathways, growth factors and other analytes have also been proposed as novel CSF biomarkers for AD [23] and the use of novel biomarkers on their own or in conjunction with traditional AD biomarkers may improve the specificity of CSF-based AD diagnosis. Recently, a proteomic approach targeting specific inflammatory and growth factors in the plasma identified novel biomarkers for the clinical diagnosis of AD [21], but the absence of pathological confirmation makes these results difficult to interpret since 10–20% of patients clinically diagnosed with AD are found on autopsy to have a cause for dementia other than AD. The determination of diagnostic accuracy for novel AD biomarkers thus requires studies of biofluids obtained during life from well-characterized AD patients longitudinally followed to autopsy confirmation [1, 3, 7]. Also, novel CSF biomarkers for AD can potentially facilitate disease staging and predict rates of clinical decline, as these biomarkers could represent factors that modulate AD pathogenesis associated with various stages of the disease. Characterization of a select panel of CSF biomarkers can, therefore, be critical in diagnosis and prognosis, and alterations in their levels can be further considered as secondary endpoints in future therapeutic trials.

Here, we tested the hypothesis that distinct sets of CSF peptides and proteins are associated with AD in contrast to cognitively normal subjects and other common neurodegenerative disorders, including FTLD with TDP-43-immunoreactive lesions (FTLD-TDP), FTLD with tau-immunoreactive inclusions (FTLD-Tau), and DLB. We collected and analyzed CSF samples antemortem from a total of 162 subjects, and the concentrations of 151 analytes in the Rules Based Medicine Human DiscoveryMAP™ panel (referred to below here as MAP) were measured by a Luminex-based multiplex platform. As the choice of analytical strategy in this type of high-dimensional data may significantly alter the composition of the identified biomarker panel, we analyzed the same body of data through three independent algorithms. Alterations in the levels of these candidate biomarkers were first analyzed according to the traditional statistical modeling, including Mann–Whitney *U* test and logistic regression. To avoid bias associated with feature pre-selection by univariate analysis and instability of linear models associated with high-dimensional data, we searched for novel AD biomarkers by two additional methods: a tree-based classification algorithm (random forest) and a nearest shrunken centroid algorithm (predictive analysis of microarrays, or PAM) [21, 29]. The diagnostic accuracy of novel analyte combinations predicted by each algorithm was then assessed. Lastly, novel AD biomarkers were then evaluated for their relationship to the severity of cognitive impairment in AD, and their potential role in predicting rates of cognitive decline in patients with mild cognitive impairment (MCI).

Materials and methods

Participants

Patients and control subjects were recruited and longitudinally followed at Penn in specialty services dedicated to the evaluation and management of neurodegenerative diseases (Supplementary Table 1). All protocols were approved by the Penn Institutional Review Board. Each patient in the autopsy cohort had undergone detailed cognitive, neurological, neuroimaging and laboratory examinations to ensure the accuracy of clinical diagnosis according to established criteria for AD [6], frontotemporal dementia (FTD) [16], amyotrophilc lateral sclerosis (ALS) [22] and DLB [13]. Autopsy-confirmed cases of AD ($n = 66$), FTLD ($n = 16$) and DLB ($n = 2$) were characterized neuropathologically with detailed immunohistochemical analysis for pathology associated with each major neurodegenerative disorder, including Aβ42, hyperphosphorylated tau, hyperphosphorylated TDP-43 and alpha-synuclein as described by Neumann et al. [18].

Seven patients with clinical FTD-ALS, but no autopsy was added to the FTLD-TDP group, as these cases nearly always have TDP-43 pathology. Thirty-eight patients with MCI were also recruited to assess predictors of cognitive decline. Each MCI patient was diagnosed by modified Petersen criteria [30], and followed longitudinally with serial cognitive and neurological examination. Cognitively normal subjects were evaluated at the time of CSF collection, and continued to undergo annual testing to confirm their cognitive status. ApoE genotyping was performed for all subjects (Supplementary Material).

Procedures

Baseline CSF samples were obtained during routine diagnostic lumbar puncture as previously described [3, 24]. Briefly, lumbar puncture was performed with a 20- or 24-gauge spinal needle, and CSF was transferred into polypropylene tubes. At the time of CSF collection, aliquots (0.5 mL) were prepared, bar-coded and then stored in polypropylene vials at −80°C until analysis (mean 8.7 years, SD = 3.6 years). Samples were then grouped altogether and simultaneously interrogated by Rules-Based Medicine, Inc. (Austin, TX) for levels of 151 analytes using the Human DiscoveryMAP™ panel and a Luminex 100 platform (Supplementary Material). The 151 MAP analytes were assembled by RBM into pre-formatted assays that RBM designed for studies of a number of different diseases including cancer, autoimmune disorders and AD based on the previous associations with AD of many, but by no means all of these analytes in peer-reviewed literature. Measures of CSF Aβ42, total tau and p-tau$_{181}$, were performed using the multiplex xMAP Luminex platform (Luminex Corp, Austin, TX) with Innogenetics (INNO-BIA AlzBio3, Ghent, Belgium) immunoassay kit-based reagents as described [24].

Statistical analysis

Statistical analysis was performed in SPSS 12.0, Random Forests (http://www.stat.berkeley.edu/~breiman/RandomForests/) and SAM/PAM. For testing of stability associated with each analyte, Pearson's correlation analysis was performed between analyte levels and time in −80°C storage. For each analytical strategy, diagnostic performance (sensitivity, specificity, accuracy) was determined using traditional AD biomarkers alone (tau, p-tau$_{181}$, Aβ42), MAP biomarkers alone or both traditional and MAP biomarkers. For each model, performance characteristics reported were based on the cross-validation. In the first model (Model 1), analytes that differed significantly between cognitively normal and AD by Mann–Whitney U test (nominal $P < 0.01$) were entered into logistic regression models for AD identification, adjusting for age and gender. Sensitivity and specificity of Model 1 were obtained by leave-one-out approach in discriminant analysis. In random forest analysis, analytes were entered into the analysis with nodes optimized for best classification of AD versus cognitively normal (Model 2). Out-of-box error rate was used to derive diagnostic accuracy, with sensitivity and specificity derived from the confusion matrix. In PAM, analytes that significantly differentiated AD from cognitively normal were identified, and diagnostic accuracy was derived through internal cross-validation (Model 3). Given the number of analytes relative to the number of subjects, interaction terms were not entered in the logistic regression model (Model 1). Random forest analysis (Model 2) and PAM (Model 3) each relies less on the assumption of normal distribution and takes into account possible correlations between analytes, although each algorithm can derive different analytes to account for variations in the respective classification model. Thus, to expand our analysis beyond the strengths and constraints of any one algorithm, we sought to identify biomarkers determined by at least two of these three well-established analytical strategies as key novel biomarkers. A similar three-approach strategy was employed to determine biomarkers that distinguished between AD and non-AD neurodegenerative disorders.

For cross-sectional association between novel AD biomarker levels and severity of cognitive impairment at the time of CSF collection, Pearson's correlation coefficient was used to relate levels of newly identified CSF AD biomarkers with cognitive performance characterized by Mini-Mental Status Examination (MMSE) in autopsy-confirmed AD cases. For correlation of CSF biomarker levels and rates of cognitive decline following CSF collection in MCI, rates of cognitive decline were first estimated by the slope of MMSE score linear regression over time. Pearson's correlation coefficient was then determined for CSF biomarker levels and rates of cognitive decline. Effects from age and gender were adjusted for all diagnostic and progression models.

Results

All CSF was obtained from patients with informed consent as described [2, 12, 24]. Levels of 151 analytes in the MAP were measured in the CSF, with 106 analytes having measurable levels for analysis (Supplementary Table 2). Four analytes (angiotensinogen, BMP-6, endothelin-1, SGOT) demonstrated level changes that corresponded to time stored in −80°C freezer and were excluded from the analysis because of their apparent instability with increasing length of storage. To determine the best biomarkers of AD, we used three independent analytical strategies to

identify MAP analytes associated with AD, and combined traditional AD biomarkers and MAP analytes to identify complementary AD biomarkers.

AD versus cognitively normal

In Model 1, 21 MAP analytes were found to differ between cognitively normal subjects and AD (Fig. 1) by Mann–Whitney *U* test at $P < 0.01$, and only a minority of these were specifically changed in AD, including resistin and thrombospondin-1. MAP analytes alone, but not traditional AD biomarkers, were entered into a forward stepwise logistic regression model (Table 1). Leave-one-out discriminant analysis using the five resultant MAP analytes achieved 84.8% sensitivity and 87.9% specificity, with overall 85.9% accuracy. By comparison, traditional AD biomarkers Aβ42 and total tau yielded greater sensitivity (92.4%), but less specificity (81.8%) for overall accuracy of 88.9%. Combining MAP analytes and traditional AD biomarkers resulted in a model differentiating AD from cognitively normal subjects by the following biomarkers: levels of tau, Aβ42, complement 3 (C3), neuron-glia-CAM-related cell adhesion molecule (NrCAM) and platelet-derived growth factor (PDGF, Table 1). This combined model has high sensitivity (97.0%) and specificity (93.9%) with 96.0% accuracy, and improved upon the traditional AD model by correctly reclassifying up to four cognitively normal subjects with pathologic CSF levels of tau and Aβ42, and three AD subjects with non-pathologic levels of CSF tau and Aβ42.

Feature pre-selection and the lack of an independent validation set may bias our classification results. Hence, we performed a similar analysis of AD versus cognitively normal through random forest (Model 2) and PAM (Model 3) using age, gender and levels of 3 traditional biomarkers and 106 MAP analytes, as each analysis incorporates internal cross-validation that is more objective than leave-one-out analysis. Model 2 using MAP analytes alone identified some analytes from Model 1, including C3, fatty acid-binding protein (Fabp), IL-23, NrCAM and PDGF, among others (Table 2; Fig. 2a). The out-of-box error rate of traditional AD biomarkers was 12.1%, which reduced to 6.1% when MAP analytes were introduced with 93.9% accuracy. Model 3 also identified NrCAM and PDGF as

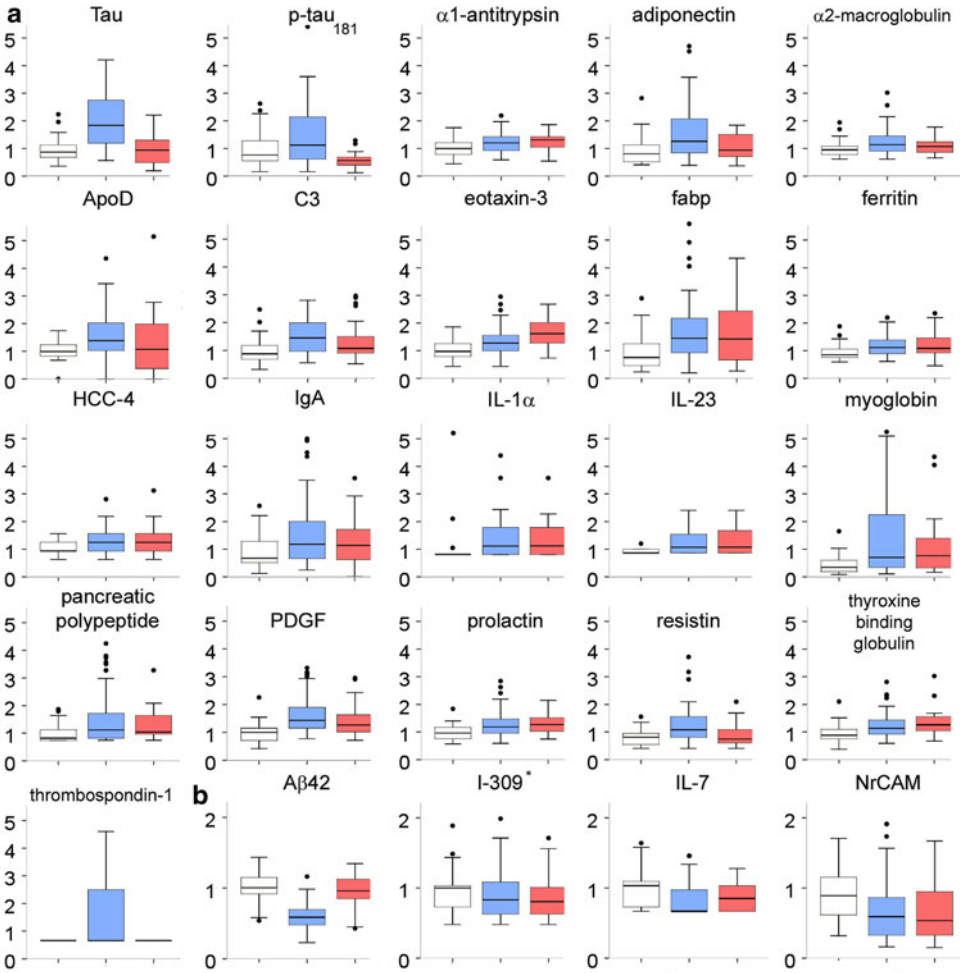

Fig. 1 Boxplots showing median values, quartiles, and outliers (*circles*) of traditional (i.e. tau and Aβ42) and other candidate CSF biomarkers that differed in levels between subjects with normal cognition and AD. Values shown are normalized to mean values of cognitively normal subjects. **a** Analytes elevated in AD as compared to cognitively normal subjects. **b** Analytes decreased in AD as compared to cognitively normal subjects. Levels in patients with autopsy-confirmed non-AD neurodegeneration were also shown for comparison. *White box* cognitively normal subjects, *blue box* autopsy-confirmed cases of AD, *red box* autopsy-confirmed cases of non-AD neurodegenerative disorders. *I-309 was found to differ between AD and cognitively normal subjects by random forest and PAM, but not Mann–Whitney *U* test

Table 1 Factors predictive of AD compared with cognitively normal subjects according to logistic regression

AD versus cognitively normal	*B*	*P*
MAP model		
Age	−0.073	0.141
Male gender	1.845	0.092
C3	0.932	0.017
Fabp	0.809	0.002
IL-23	17.24	0.031
NrCAM	−0.051	<0.001
PDGF	0.004	0.064
Traditional AD model		
Age	−0.024	0.558
Male gender	0.001	0.999
Aβ42	−0.035	<0.001
Tau	0.019	0.051
Combined model		
Age	−0.217	0.088
Male gender	2.038	0.309
C3	2.376	0.025
NrCAM	−0.063	0.041
PDGF	0.013	0.061
Tau	0.08	0.042
Aβ42	−0.039	0.01

Traditional AD model incorporated Aβ42 and tau levels

Coefficient (*B*) and *P* value for each factor as part of the overall model are shown

Age and gender were entered into first block of LR, while analytes identified to be different between AD and cognitively normal subjects were then entered in a forward step-wise fashion, with $P < 0.05$ for entry and $P > 0.10$ for removal

Table 2 Analytes differentiating AD from cognitively normal according to random forest analysis

AD versus cognitively normal	*Z* score
MAP model	
PDGF	27.585
IL-1α	26.656
IL-23	20.686
C3	18.343
Fabp	17.888
NrCAM	15.937
VEGF	13.894
TRAIL-R3	12.064
IL-17	11.209
Eotaxin-3	11.037
IL-7	10.48
A2M	9.603
Prolactin	9.549
Ferritin	8.463
ThBG	8.371
I-309	7.743
HCC-4	3.502
Traditional AD model	
Aβ42	68.401
Tau	21.826
Male gender	10.052
Combined model	
Aβ42	47.935
Tau	26.232
PDGF	22.506
IL-1α	19.806
NrCAM	17.474
C3	16.702
IL-23	14.472
VEGF	13.362
Fabp	11.583
Prolactin	11.533
TRAIL-R3	9.779
A2M	9.425
I-309	7.981
Calcitonin	5.824
Adiponectin	4.64

important biomarkers useful in distinguishing between AD and cognitively normal subjects (Tables 3, 4; Fig. 2a). Diagnostic accuracy obtained through cross-validation was 93.9% in Model 3. A summary of analytes important in distinguishing between AD and cognitive normal subjects is shown in Fig. 2a, including Aβ42, tau, NrCAM and PDGF identified by all three algorithms.

AD versus other neurodegenerative disorders

With the emergence of substrate-specific therapeutic interventions, it is critically important to identify biomarkers that reliably differentiate the major neurodegenerative disorders from one another. To this end, we assessed which CSF biomarkers best differentiated AD from other neurodegenerative disorders using a similar series of analytical strategies.

Among traditional AD biomarkers, Aβ42 and p-tau$_{181}$ levels discriminated between AD and non-AD neurodegenerative disorders in all models. Among novel MAP analytes, agouti-related peptide (AgRP) was identified by all algorithms to distinguish between AD and non-AD disorders (Fig. 2b). Post hoc analysis showed AgRP as most altered in FTLD-TDP (Fig. 3) and its classification power may rest in identifying FTLD-TDP cases. Tau, eotaxin-3 and hepatocyte growth factor (HGF) were additionally identified by both RF and PAM to be important in distinguishing between AD and non-AD disorders (Fig. 2b). Similar to the classification role of AgRP, eotaxin-3 was most different between AD and FTLD-TDP ($P = 0.001$), and HGF was most different between AD and

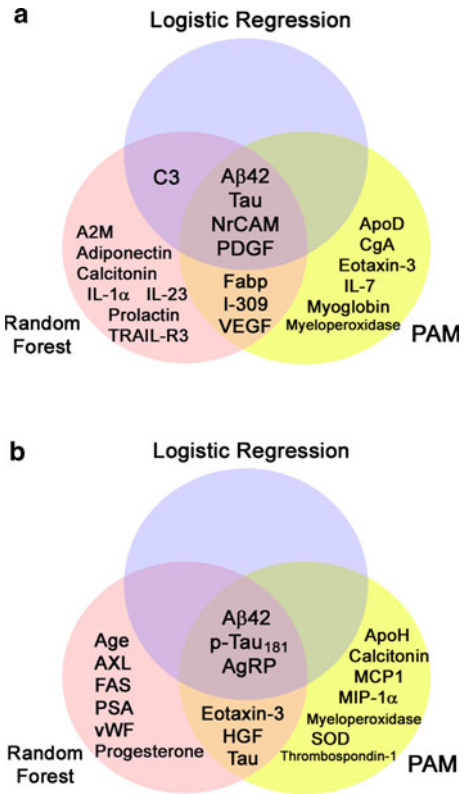

Fig. 2 AD biomarkers identified by each of the three analytical strategies (logistic regression, random forest, and PAM). **a** Biomarkers useful in distinguishing between subjects with AD and normal cognition. **b** Biomarkers useful in distinguishing between subjects with AD and other non-AD neurodegenerative disorders. Analytes in overlapping regions were identified by multiple strategies as important biomarkers

FTLD-Tau ($P = 0.002$, both comparisons by Mann–Whitney U test; Fig. 3). Thus, biomarkers more specifically associated with other neurodegenerative disorders can also aid in the diagnosis of AD.

Biomarker associations with cognitive function and decline

Some diagnostic biomarkers may reflect severity of cognitive impairment and thus be useful in disease staging. To assess this, we correlated CSF biomarker levels with MMSE scores at the time of CSF collection as a general measure of cognitive impairment. Among CSF biomarkers for AD identified by at least one approach, six (C3, CgA, IL-1α, I-309, NrCAM and VEGF) were correlated with MMSE score, and levels of these analytes did not correlate with MMSE scores in the other neurodegenerative disorders. A multivariate linear regression analysis adjusting for age, gender and education showed C3, IL-1α and I-309 levels were independently associated with MMSE scores in autopsy-confirmed cases of AD.

Table 3 Factors differentiating AD from cognitively normal according to PAM

MAP model	Traditional AD model	Combined model
AD versus cognitively normal		
PDGF	Aβ42	Aβ42
VEGF	Tau	Tau
NrCAM	p-tau$_{181}$	PDGF
CgA	Age	VEGF
ApoD		NrCAM
Fabp		CgA
I-309		ApoD
Eotaxin-3		Fabp
IL-7		Eotaxin-3
Myoglobin		I-309
Myeloperoxidase		IL-7
GRO-α		Myoglobin
EN-RAGE		Myeloperoxidase
TGFα		
Thrombospondin-1		
Age		
Stem cell factor		
Tissue factor		
Pancreatic polypeptide		
MDC		
TECK		
SOD		
Ferritin		
EGF-R		
IL-11		
FAS		
IL-1ra		
Prolactin		
AXL		
IL-17		
TRAIL-R3		
FAS-ligand		
IL-16		

Threshold was set for each model through internal cross-validation

To further test the value of these CSF biomarkers in predicting cognitive decline, we determined the correlation between levels of these six biomarkers and rates of subsequent MMSE decline in MCI subjects following CSF collection. The 38 living MCI patients were similar to AD patients in age (71.39 vs. 70.79 yo, $P = 0.674$), education (15.66 vs. 14.64 yo, $P = 0.143$) and gender (42.1 vs 53.0% women), but MCI patients had higher MMSE scores (mean 26.16, SD = 2.00) as compared to AD (mean 17.55, SD = 8.57, $P < 0.01$). The MCI patients had a median follow-up of 52 months (range 30–129 months) and a

Table 4 Comparative diagnostic performance of biomarker panels according to random forest versus PAM analysis for AD versus cognitively normal

	Random forest			PAM		
	Traditional	MAP	Combined	Traditional	MAP	Combined
Sensitivity (%)	88.6	86.4	92.4	97.0	89.4	97.0
Specificity (%)	86.2	81.8	97.0	66.7	75.8	87.9
Accuracy (%)	87.9	84.8	93.9	86.9	84.8	93.9

Traditional models included established AD biomarkers (Aβ42, tau and/or p-tau$_{181}$). MAP models included novel biomarkers from the MAP 151 analyte panel. Combined models included traditional and MAP biomarkers

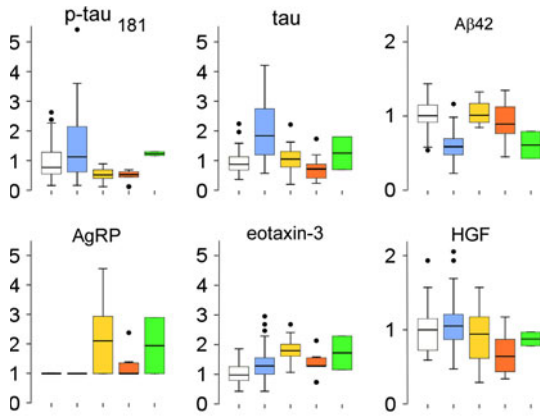

Fig. 3 Boxplots showing median values, quartiles, and outliers (*circles*) of traditional and candidate biomarkers that differed in levels between AD and other non-AD neurodegenerative disorders. Values shown are normalized to mean values of cognitively normal subjects. *White box* cognitively normal subjects, *blue box* AD, *yellow box* FTLD-TDP, *orange box* FTLD-Tau, *green box* dementia with Lewy bodies

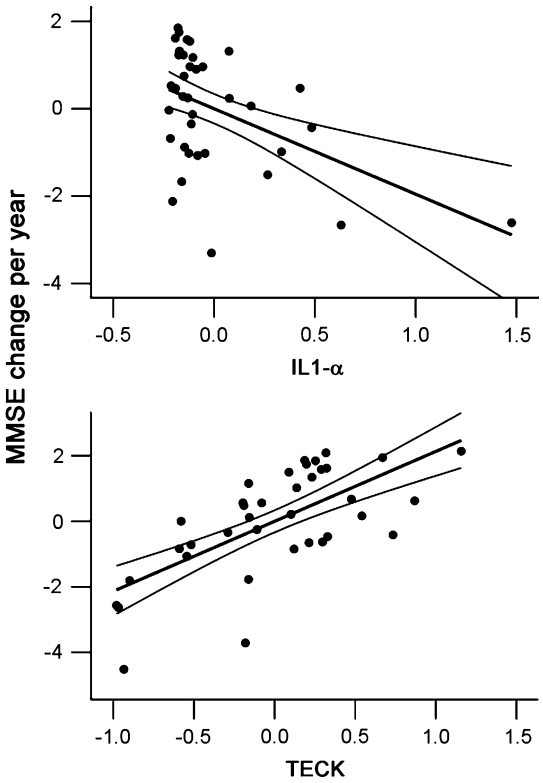

Fig. 4 Partial residual plots of MAP analytes versus rates of subsequent cognitive decline in MCI. Linear fit and 95% confidence interval for fit are shown for each graph. The overall model includes age, gender, education, IL-1α level and TECK level

median rate of MMSE decline of 1.2 points per year (mean 2.0, SD = 2.0). Among analytes associated with cognitive performance at the time of CSF collection in AD, IL-1α levels correlated with the rates of MMSE decline ($P = 0.003$), although with modest effect on decline rates ($R = 0.498$ for model). A search across 4 traditional and 106 MAP analytes additionally identified thymus-expressed chemokine (TECK) as significantly associated with rates of cognitive decline in MCI ($P < 0.001$ adjusting for age, gender and education) and had a stronger effect on the rate of decline ($R = 0.745$ for model, Fig. 4).

Discussion

The search for accurate CSF and plasma biomarkers in neurodegenerative diseases has intensified with the increasing need for informative biomarkers in clinical trials of disease-modifying therapies for AD, and has been facilitated by high-throughput multiplex platforms [21, 24]. Using clinically and pathologically well-characterized cases of AD and FTLD, we identified novel biomarkers useful in improving the distinction between AD and cognitively normal subjects, such as NrCAM and PDGF and biomarkers associated with other disorders that improved the classification between AD and non-AD dementia, including AgRP, eotaxin-3 and HGF. C3, IL-1α and I-309 were helpful in the staging of AD, and IL-1α and TECK levels correlated with rates of subsequent cognitive decline in MCI. We discuss these findings below.

In none of our analytical models did MAP biomarkers alone out-perform traditional AD biomarkers in

identifying AD from non-AD cases, but they complemented traditional biomarkers in two ways. First, while decreased Aβ42 and increased total/phosphorylated-tau levels are strongly linked to AD, altered levels of some MAP biomarkers improved the classification of cognitively normal subjects with decreased levels of CSF Aβ, but no dementia. Alterations in MAP biomarkers (NrCAM, PDGF) were seen in multiple neurodegenerative disorders, and likely represent neuronal loss rather than AD-specific processes (Fig. 1). NrCAM is a member of the L1 family of cell adhesion molecules, and may be involved in ion channel clustering at the axon initial segment and nodes of Ranvier [4, 5]. A decrease in NrCAM levels in AD and other neurodegenerative disorders likely follows axonal degeneration, although accumulation of ankryn (which interacts with NrCAM intracellularly) in the insoluble AD proteome relative to normal and FTLD-TDP brains raises additional possibilities [10]. PDGF was previously identified as a plasma AD biomarker by Ray et al. [21]. PDGF-receptor activation can promote Aβ precursor protein processing in vitro [9], and inhibition of PDGF-receptor activation with imatinib mesylate can decrease Aβ40 and Aβ42 secretion [17]. In our cohort, PDGF was also found to be elevated in multiple disorders, and its constitutive expression by neurons [8] suggests elevated PDGF levels to also reflect more general neuronal loss. C3 and Fabp were identified as AD biomarkers by two algorithms, and CSF Fabp was elevated in AD and DLB cases in one other study [25]. Despite alterations in multiple forms of dementia, however, changes in these biomarkers associated with neuronal loss improved the distinction between AD and cognitively normal with age-associated amyloidosis by traditional AD biomarkers alone, and they can further serve as secondary endpoints in therapies aimed at Aβ42 or tau clearance.

In addition to traditional AD CSF biomarkers (i.e. tau and Aβ42), altered levels of resistin and thrombospondin-1 specifically associated with AD despite little classification value beyond analytes in Fig. 2a. Central resistin modulates leptin action and oral intake [19], and resistin as a marker of macrophage may mean preferential microglial activation. Thrombospondin-1 is a key molecule in astrocyte-induced neurogenesis [14], and can promote recovery after brain ischemia. Elevated thrombospondin-1 levels in a subgroup of AD patients may identify a unique subgroup with vascular and degenerative etiologies for their dementia. Further stratification of AD patients by their CSF resistin and/or thrombospondin-1 levels in a larger cohort should clarify their role in AD lesions.

Novel MAP biomarkers also represent candidate biomarkers of disease staging and prediction of progression. Cross-sectionally, six diagnostic AD biomarkers correlated with cognitive deficits at the time of CSF collection. Because these analytes likely mirror severity of neurodegeneration, correlations between their levels and clinical status should be expected. Furthermore, IL-1α levels were modestly associated with rates of decline in MCI after the CSF was collected. IL-1α immunoreactive microglia in AD neuritic plaques have been implicated in plaque evolution [11], and the difference in IL-1α levels between fast and slow MCI decliners may signal cognitive deficits insensitive to MMSE alone. We also identified TECK to predict the rate of cognitive decline among MCI patients, even though TECK itself was not a robust diagnostic biomarker for AD. This can be due to the potential pathologic heterogeneity of MCI, or represent a biomarker change that is transient in nature and specifically linked to the MCI or pre-AD stage. TECK (CCL25) is best understood as a strong chemoattractant for thymocytes and intestinal T cells [15]. TECK is a ligand to CCR9 which is predominantly expressed in epithelial tissues, but also a ligand to CCX-CKR found in the human brain [27, 31]. The role of TECK in AD has never been investigated, and its role as a robust predictor of cognitive decline in MCI should prompt further examination of its role in AD.

Some analytes were identified by only one analytical strategy as a potential AD biomarker due to the non-uniqueness of multiple analytical strategies, begging the question of whether such analytes are "true" biomarkers. Notably, the number of ApoE4 alleles was only identified by one analytical strategy (logistic regression, data not shown) to be a significant predictor of AD versus cognitively normal, despite its known association with AD[23]. The ordinal nature of allele dosage (such as number of ApoE4 alleles) may be more suited for models using linear scaling and less preferred by random forest and PAM. Consequently, we elected to seek analytes of wider ranging levels as novel biomarkers for more uniformity among the algorithms. Among other analytes identified only by one algorithm, IL-1α appears to be important in disease staging, and HGF was previously found to differentiate between AD and PSP [28]. Several explanations are possible. First, some analytes may correlate strongly with others, and each strategy may select different proxy analytes to represent a group of correlated analytes from the same biological process. Second, different analytical strategies may have various strengths and weaknesses for detecting particular effects. This was the reason we chose three analytical strategies to identify putative AD biomarkers, and analytes identified by multiple strategies may be most reliable. Third, some analytes identified by only one analytical strategy may be associated with chance difference at the population level not directly associated with dementia or AD. These speculations notwithstanding,

each putative novel biomarker's value in diagnosis and prognosis needs independent validation in another single- or a multicenter study, and their biological significance should be assessed independently. Indeed, we have studies underway now to do exactly this.

In summary, we identified novel biomarkers associated with pathologically confirmed AD. Some analytes were specifically associated with AD including Aβ42, resistin, and thrombospondin-1, while others were associated with multiple neurodegenerative disorders. Some diagnostic biomarkers mirrored the severity of cognitive impairment at time of CSF collection, while TECK and IL-1α reflected the rate of cognitive decline among clinically diagnosed MCI subjects. Accordingly, we propose the inclusion of diagnostic and prognostic biomarkers in a composite AD biomarker panel. Given the variability of each candidate biomarker across individuals, their collective classifying power should be determined in a large multicenter cohort, such as the Alzheimer Disease Neuroimaging Initiative. The biological relevance of each individual and set of biomarkers should be investigated for potential targets of therapeutic developments.

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