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CSF peptides from VGF and other markers enhance prediction of MCI to AD progression using the ATN framework

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ABSTRACT

The amyloid beta, tau, neurodegenerative markers framework has been proposed to serve as a system to classify and combine biomarkers for Alzheimer's Disease (AD). Although cerebrospinal (CSF) fluid AT (amyloid beta and tau)-based biomarkers have a well-established track record to distinguish AD from control subjects and to predict conversion from mild cognitive impairment (MCI) to AD, there is not an established non-tau based neurodegenerative ("N") marker from CSF. Here, we examine the ability of several candidate peptides in the CSF to serve as "N" markers to both classify disease state and predict MCI to AD conversion. We observed that although many putative N markers involved in synaptic processing and neuroinflammation were able to, when examined in isolation, distinguish MCI converters from non-converters, a derivative from VGF, when combined with AT markers, most strongly enhanced prediction of MCI to AD conversion. Low CSF VGF levels were also predictive of MCI to dementia conversion in the setting of normal AT markers, suggesting that it may serve as a very early predictor of dementia conversion. Other markers derived from neuronal pentraxin 2, GAP-43 and a 14-3-3 protein were also able to enhance MCI to AD prediction when used as a marker of neurodegeneration, but VGF had the highest predictive capacity. Thus, we propose that low levels of VGF in CSF may serve as "N" in the amyloid beta, tau, neurodegenerative markers framework to enhance the prediction of MCI to AD conversion.

1. Introduction

Alzheimer's Disease (AD) is growing in prevalence with the aging of the population. Approximately 10% of individuals over the age of 65 meet criteria for AD, and by 2050, it is expected that 152 million individuals worldwide will have AD (GBD Dementia Forecasting Collaborators, 2022). Although a cure for this disease does not exist, it is likely that potential disease-modifying therapies will have their largest impact in the earliest stages of the illness. Since potential disease-modifying therapies, such as amyloid reduction approaches, may carry risks, it is imperative that we have tools to

Abbreviations: Aβ, amyloid beta; AD, Alzheimer Disease; BEN, Bayesian Elastic Net; CHI3L1, Chitinase 3 Like 1; CSF, cerebrospinal fluid; CUI, cognitively unimpaired; EMCI, early MCI; GAP-43, growth-associated protein-43; LMCI, late MCI; MCI, mild cognitive impairment; MRI, magnetic resonance imaging; NL, normal subject; NPTX2, neuronal pentraxin-2; PET, positron-emission tomography; RLL, Resampled Logistic Lasso; RRF, Regularized Random Forests; p-tau, phosphorylated tau at site 181; SGB, Stochastic Gradient Boosting; SMC, subjective memory complaint; SPP1, Osteopontin; t-tau, total tau; YWHAZ, 14-3-3 protein zeta/delta.

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[#] Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

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identify and select individuals at the earliest stages of this illness with the highest likelihood for progression so that we can provide such therapeutic interventions to those with the highest capacity to benefit.

To that end, multiple biomarkers have been proposed as stratification tools for AD. Recently, a framework has been proposed to classify and combine biomarkers, known as the "ATN" framework (Jack et al., 2018; Jack et al., 2016). In this system, "A" corresponds to markers of amyloid beta ($A\beta$) deposition, such as accumulation of $A\beta$-binding radioligand on a PET scan or depressed levels of various $A\beta$ species in cerebrospinal fluid (CSF). "T" corresponds to accumulation of various species of tau protein, commonly measured as tau-binding radioligand on a positron-emission tomography (PET) scan or elevation of phosphorylated tau in CSF. It is well established that, although most AD patients express abnormalities in both $A\beta$ and tau, A and T are not redundant markers, and their levels can be combined to provide enhanced detection of AD and prediction of conversion from mild cognitive impairment (MCI) to AD. "N" corresponds to markers of neurodegeneration. The most common metric of neurodegeneration is regional brain atrophy, typically of the hippocampus or CSF total tau. Again, though most AD patients have abnormalities in all of these markers, they are not entirely overlapping, thus can be combined (A+T+N) to provide additional power to detect the earliest stages of AD pathology.

Unfortunately, except in the case of using CSF total tau as a marker of neurodegeneration (addressed below), current approaches to measure A+T+N require multimodality and expensive technology to measure all of these markers. For example, an individual would need to undergo CSF analysis + quantitative high resolution MRI, or high resolution structural MRI + amyloid + tau PET scans (both of which are not yet covered by most U.S. insurers) to have all 3 markers quantified. Thus, identification of an additional "N" marker in the CSF, to be combined with AT would mark a significant advancement, and would expose the patient to a single relatively inexpensive test to classify patients and to assess their risk for progression from MCI to AD. We have previously demonstrated that novel CSF peptide markers of neurodegeneration, either on their own or when combined with CSF amyloid/tau markers within the ATN framework, have significant diagnostic and prognostic utility in AD diagnosis and progression (Devanarayan et al., 2019; Llano et al., 2017; Llano et al., 2019). In addition, recent data from specific peptide sequences/forms of 5 proteins (Chitinase 3 Like 1, Neuronal Pentraxin-2, Osteopontin, VGF, and 14-3-3 protein zeta/delta) using targeted proteomics by mass spectrometry in CSF were made available for 719 subjects in the ADNI database (Watson et al., 2021). These proteins were previously identified by integrative proteomics as brain-based CSF biomarkers modulated in patients with AD (Higginbotham et al., 2020). In our own prior work cited above, we reported other peptide sequences of VGF and a peptide from a protein that is closely related to neuronal pentraxin 2 (the neuronal pentraxin receptor, NPTXR) to be highly significant and predictive markers of AD diagnosis and progression (Devanarayan et al., 2019; Llano et al., 2019).

Thus, in this report, we study the potential utility of these proteins in their specific peptide forms for AD diagnosis and progression via machine-learning algorithms in a novel patient population compared to previous analyses. In this analysis, we also include data from the full protein form of growth associated protein-43 (GAP-43) on these same Alzheimer's Disease Neuroimaging Initiative (ADNI) subjects (data made available by Sandelius et al., 2019) as it has been reported to be specific to AD and associated with tau and amyloid pathology (Sandelius et al., 2019). We study the collective utility of these 6 proteins, both on their own and in combination with CSF amyloid/tau markers, within the ATN framework (Jack et al., 2018; Jack et al., 2016) to both classify disease state and predict conversion from MCI to AD.

2. Methods

2.1. Database

Data used in the preparation of this article were obtained from the ADNI database (adni.loni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), PET, other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early AD. For up-to-date information, see www.adni-info.org. This study was registered under clinicaltrials.gov under ClinicalTrials.gov Identifier: NCT00106899. The study was conducted across multiple clinical sites and was approved by the Institutional Review Boards of all of the participating institutions. Informed written consent was obtained from all participants at each site. The following individual ethics boards approved the study: Albany Medical College Institutional Review Board, Boston University Medical Campus Institutional Review Board (BU IRB), Butler Hospital Institutional Review Board, Cleveland Clinic Institutional Review Board, Columbia University Institutional Review Board, Dartmouth-Hitchcock Medical Center Committee for the Protection of Human Subjects, Duke University Health System Institutional Review Board, Emory University Institutional Review Board Georgetown University Institutional Review Board, Human Investigation Committee Yale University School of Medicine, Human Subjects Committee, University of Kansas Medical Center, Indiana University Institutional Review Board, Research Compliance Administration, Institutional Review Board of Baylor College of Medicine, Institutional Review Board of the Mount Sinai School of Medicine, Johns Hopkins University School of Medicine Institutional Review Boards, Lifespan—Rhode Island Hospital Institutional Review Board, Mayo Clinic Institutional Review Board, Nathan Kline Institute Rockland Psychiatric Center Institutional Review Board (NKI RPC IRB), New York University Langone Medical Center School of Medicine, Institutional Review Board Human Research Program, Northwestern University Institutional Review Board Office, Office of the Washington University School of Medicine IRB (OWUMC IRB), Oregon Health and Science University Institutional Review Board, Partners Human Research Committee, Research Ethics Board Jewish General Hospital, Research Ethics Board Sunnybrook Health Sciences Centre, Roper St. Francis Institutional Review Board, Rush University Medical Center Institutional Review Board, Stanford University, Administrative Panel on Human Subjects in Medical Research, The Ohio State University Institutional Review Board, The University of Texas Southwestern Medical Center Institutional Review Board, UCLA Office of the Human Research Protection Program Institutional Review Board, UCSD Human Research Protections Program, University Hospitals Case Medical Center Institutional Review Board, University of Alabama at Birmingham Institutional Review Board, University of British Columbia, Clinical Research Ethics Board (CREB), University of California Davis Office of Research IRB Administration, University of California Irvine Office Of Research Institutional Review Board (IRB), University of California San Francisco Committee on Human Research (CHR), University of Iowa Institutional Review Board, University of Kentucky Office of Research Integrity, University of Michigan Medical School Institutional Review Board (IRBMED), University of Pennsylvania Institutional Review Board, University of Pittsburgh Institutional Review Board, University of

Rochester Research Subjects Review Board (RSRB), University of South Florida Division of Research Integrity and Compliance, University of Southern California Health Science Campus Institutional Review Board, University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB), University of Wisconsin Health Sciences Institutional Review Board, Wake Forest University Institutional Review Board, Weill Cornell Medical College Institutional Review Board, Western Institutional Review Board and Western University Health Sciences Research Ethics Board. Data used for the analyses presented here were accessed on December 15, 2021. ADNI ID numbers for all subjects in the study are available in Supplemental Table 1.

2.2. Clinical diagnosis

Subjects in this study were drawn from ADNI-GO and ADNI-2, and details can be found at (ADNI-GO: http://adni.loni.usc.edu/wp-content/uploads/2008/07/ADNI_GO_Procedures_Manual_06102011.pdf, ADNI-2: http://adni.loni.usc.edu/wp-content/uploads/2008/07/adni2-procedures-manual.pdf). Entry criteria stipulate that subjects range from age 55–90 years in age, have a Hachinski score less than or equal to 4 and a Geriatric Depression Scale score less than 6. As described in Aisen et al. (Aisen et al., 2015), early MCI (EMCI) patients had a memory complaint, an abnormal score on the Logical Memory II subscale from the Wechsler Memory Scale-revised, with a score of 9–11 with 16 years of education, 5–9 with 8–15 years of education or 3–6 with 0–7 years of education (corresponding to a Z score of approximately -1.0). Those with late MCI (LMCI) had corresponding Logical Memory II subscale scores of $\leq$8, $\leq$4 or $\leq$2 (based on education level, corresponding to a Z score of approximately -1.5). All MCI subjects had MMSE scores between 24-30 and a Clinical Dementia Rating scale score of 0.5. AD subjects had similar entry criteria as LMCI, but had MMSE scores between 20 and 26 (inclusive) and CDR scores of 0.5 or 1.0 and met NINCDS/ADRDA criteria for probable AD (McKhann et al., 1984). Subjects with subjective memory complaints (SMC) had a memory complaint and a score of 16 or greater on the first 12 questions on the cognitive change index. Normal subjects (NL) did not have a memory complaint, had education-adjusted scores on Logical Memory Testing of $\geq$9, $\geq$5, or $\geq$3 (per above) and had a Clinical Dementia Rating scale score of zero.

2.3. ADNI proteomics analysis of CSF by mass spectrometry

CSF was obtained at baseline for all subjects in this study. As described in ADNI study document from Watson, Seyfried, and Levey (Watson et al., 2021), a subset of CSF samples from ADNI cohort using targeted proteomics by mass spectrometry were obtained for 6 protein targets (Chitinase 3 Like 1 [CHI3L1.IASNTQSR], Neuronal Pentraxin-2 [NPTX2.VAELEDEK], Osteopontin [SPP1.QETLPSK and SPP1.GDSVVYGLR], VGF [VGF.EPVAGDAVPGPK], and 14-3-3 protein zeta/delta [YWHAZ.VVSSIEQK]). These 6 peptides were previously identified by integrative proteomics as brain-based CSF biomarkers and reported to change in patients with AD (Higginbotham et al., 2020). CSF proteins were reduced, alkylated, denatured, and enzymatically digested with Lys-C and trypsin (1:100 enzyme:protein ratio) and analyzed as a single replicate over 9 days using a standard flow Agilent 1290 Infinity II liquid chromatography system coupled with Thermo Fischer Scientific TSQ Altis Triple Quadrupole mass spectrometer at Emory University School of Medicine (Watson et al., 2021). Total area ratios are reported for each peptide.

To confirm the relationship between depressed VGF levels and conversion from MCI to AD in subjects with normal ratios of t-tau to Aβ1-42, we examined levels of a VGF peptide (VGF_NSEPQDEGELFQGVDPR) in an independent cohort from the ADNI dataset. This peptide was measured using multiple reaction monitoring (MRM) mass spectrometry. Please see (Devanarayan et al., 2019) for details.

2.4. ADNI CSF GAP-43

GAP-43 in CSF samples was analyzed using enzyme-linked immunoassay. The GAP-43 analyses were performed at the Clinical Neurochemistry Lab by a board-certified laboratory technician at the University of Gothenburg, Sweden. Mouse monoclonal GAP-43 antibody NM3 (coating antibody) and a polyclonal GAP-43 antibody (detector antibody) were used to recognize the C-terminal of GAP-43. A total of 18 ELISA plates were analyzed with an assay range of 312 – 20,000 pg/mL in 4 analytical runs to result in 1268 data points (Sandelius et al., 2019).

2.5. Data analysis

2.5.1. Univariate data analysis

Significance of each of the markers in relation to disease state (NL vs. SMC vs. EMCI vs. LMCI vs. AD) was assessed within the framework of analysis of covariance after adjusting for age, gender, body mass index (BMI), and education as covariates. All markers were log transformed, and multiplicity adjustments for the comparison of disease states to NL were made via Dunnet's method. Similar analysis method was also applied for assessing the association of each of these markers at baseline with the subsequent 36-month progression of the EMCI and LMCI subjects to AD.

2.5.2. Multivariate data analysis

The predictive modeling to derive signatures based on these markers to classify the cognitively unimpaired (CUI; NL + SMC) subjects and AD subjects was carried via different machine-learning algorithms such as Resampled Logistic Lasso (RLL), Bayesian Elastic Net (BEN), Regularized Random Forests (RRF) and Stochastic Gradient Boosting (SGB). RLL and BEN assume linear relationships of the markers with the disease state odds, whereas RRF and SBG are tree-based ensemble methods that take into consideration of any inherent nonlinear relationships between the markers versus disease state odds and their interactions via a data-driven manner (not requiring specification of the specific relationships and interactions). Performance of the signatures derived from these algorithms for accurately classifying CUI versus AD subjects was estimated via 10 iterations of 10-fold stratified cross-validation (see details in (Devanarayan et al., 2019)).

As the signatures from SGB outperformed the signatures from the other algorithms based on this cross-validation, results from only the SGB are reported in this paper. These signatures were then applied to an independent group of EMCI and LMCI subjects at baseline to predict their future progression to AD within 36-months. The EMCI and LCMI subjects that were predicted at baseline to be AD-like (called "Signature Positive") were considered as future converters to AD, and those predicted to be NL-like (called "Signature Negative") were considered as non-converters. These baseline predictions of the MCI subjects were then compared to the follow-up clinical data. Performance metrics such as the sensitivity, specificity and balanced accuracy were calculated by comparing the predictions to the known progression status of the MCI subjects to AD over the next 36-months.

These multivariate signatures were then evaluated for their ability to differentiate the future time to progression of the EMCI

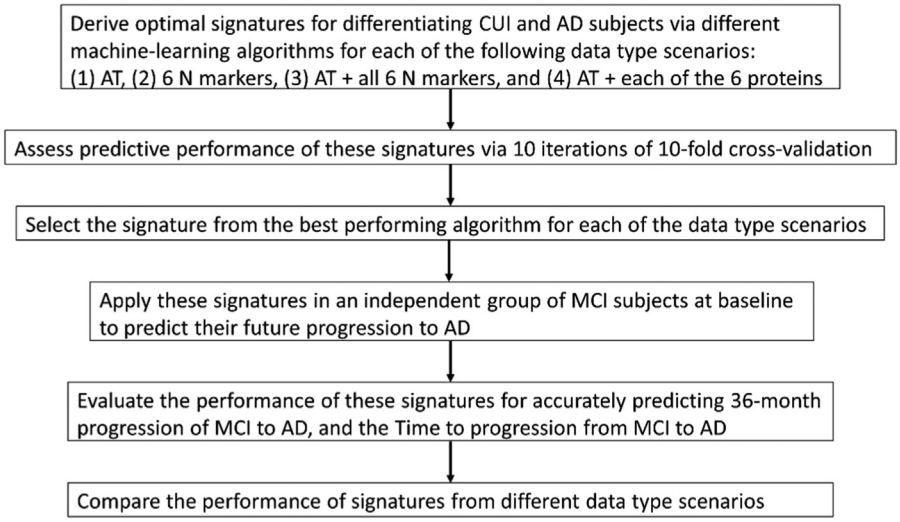

Fig. 1. Predictive modeling flow scheme for deriving multivariate signatures of disease diagnosis and prognostic prediction of MCI to AD progression. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and LMCI subjects to AD. This was accomplished by comparing the time to AD progression of the predicted signature positive EMCI and LMCI subjects at baseline (i.e., the EMCI and LMCI subjects that were predicted to be AD-like at baseline) versus the predicted signature negative EMCI and LMCI subjects at baseline via Kaplan-Meier analysis. For this evaluation, the progression to AD over the future time course until the last follow-up visit (up to 120 months) was taken into consideration. This analysis procedure to derive an optimal signature for disease state differentiation and prediction of future progression of MCI subjects to AD was carried out separately for the following subsets of markers, along with APOE genetic status, age, gender, and education:

- AT
- 6 N proteins or peptides
- AT + all 6 N proteins or peptides
- AT + each of the 6 proteins or peptides

The optimal signature derived for each of the above scenarios from the machine-learning algorithms would include one or more of the markers from these scenarios. Please see Fig. 1 for a diagram depicting the analysis flow scheme.

As explained in (Devanarayan et al., 2019), while it is not necessary for a signature that differentiates AD versus CUI subjects to predict the progression of EMCI and LMCI subjects to AD, it is important that a signature that predicts disease progression to also be relevant for disease diagnosis as it would better reflect the AD pathology. Most importantly, this evaluation of the AD versus CUI signature on the MCI subjects at baseline to predict their future progression to AD not only served as an independent verification of the utility of the identified signatures, but also put it to a greater test to see whether it could address a different question related to the prognostic prediction of future progression of MCI subjects. All analyses related to predictive modeling and signature derivation were carried out using R (http://www.R-project.org), version 4.1.1.

3. Results

3.1. Demographics

Data from 719 subjects across NL, SMC, EMCI, LMCI, and AD were included in this study and their key demographic variables (gender, ApoE4 status, age, education, BMI and MMSE) are shown in Table 1. All of these characteristics were significantly different across two or more of these groups (in all cases $p < 0.05$, Kruskal-Wallis test used for age, education, BMI and MMSE, and Chi-Squared test used for gender and ApoE4 status). Age was not significantly different between CUI and AD. All demographic variables, except ApoE and MMSE, were adjusted for in the univariate analysis of the ATN markers as described above. Table 2 contains demographic variables for individuals in the two MCI groups (EMCI and LMCI), separated by whether they remained stable over 36-months, or converted to AD. As shown, significant predictors of conversion over 36 months were ApoE4 status (EMCI: $p < 0.0001$, LMCI: $p = 0.0015$, Chi-Squared) and MMSE for the LMCI group (EMCI: $p = 0.132$, LMCI: $p < 0.0001$, Wilcoxon rank sum test).

3.2. Correlations between the ATN markers

Spearman correlations between levels of A, T and putative N markers were examined. As expected, CSF Aβ1-42 levels were inversely correlated with total tau (t-tau, ρ = -0.474, $p < 0.0001$) and phospho-tau 181 (p-tau, ρ = -0.474, $p < 0.0001$) levels (Fig. 2). Among the putative N markers, the strongest correlations were seen between the 2 SPP species ($\rho = 0.832$, $p < 0.0001$), as well as NPTX2 and VGF ($\rho = 0.741$, $p < 0.0001$). GAP-43 and YWHAZ also strongly correlated with each other ($\rho = 0.631$, $p < 0.0001$) as well as t-tau (correlation coefficients = 0.745 and 0.715, respectively, both $p < 0.0001$), as has been previously reported (Sjögren et al., 2001; Sjögren et al., 2000; Zhou et al., 2020), suggesting that these two markers may serve as general indicators of neuronal damage. In addition, VGF and NPTX2-derived peptides had relatively low correlations with Aβ1-42 and p-tau (ρ values ranging from 0.164 to 0.282) suggesting that these may add more complementary value when combined with AT than the other markers.

3.3. CSF ATN markers across diagnostic categories

As expected, AT markers varied across diagnostic categories, as shown in Fig. 3. When compared to CUIs, all cognitively impaired groups (EMCI, LMCI, and AD) had lower CSF Aβ1-42 levels, higher p-tau and t-tau levels and higher ratios of both p-tau/Aβ1-42 and t-tau/Aβ1-42 ($p < 0.0001$ across all categories). Differences relative to CUI were progressively greater for EMCI, LMCI, and AD subjects,

Table 1
Demographics and related information on subjects from different control and disease states.

		NL	SMC	EMCI	LMCI	AD	*p*-value
Number of subjects (n)		142	83	240	141	113	
Gender (n)	**F**	75	51	108	65	45	0.0217
	M	67	32	132	76	68	
ApoE (n)	**non-E4**	106	55	137	59	38	< 0.0001
	E4	36	28	103	82	75	
Age; years, Mean (SD)		73.5 (6.3)	72.3 (5.6)	70.7 (7.5)	71.9 (7.6)	74.3 (8.3)	*< 0.0001**
Education; years, Mean (SD)		16.6 (2.5)	16.6 (2.5)	16.0 (2.6)	16.7 (2.6)	15.7 (2.7)	0.0033
BMI; Mean (SD)		27.3 (4.4)	28.2 (5.8)	28.2 (5.3)	27.2 (4.8)	26.0 (5.1)	0.0014
MMSE; Mean (SD)		29.1 (1.2)	29.0 (1.1)	28.4 (1.6)	27.6 (1.9)	23.2 (2.0)	< 0.0001

Table 2
demographics and related baseline information on EMCI and LMCI subjects, stratified by their conversion status to AD over 36 months.

		EMCI		LMCI		p-value (Stable vs. Conversion)	
		Stable	Converter	Stable	Converter	EMCI	LMCI
Number of subjects (n)		168	19	63	56		
Gender (n)	**F**	75	5	28	26	0.1168	0.8282
	M	93	14	35	30		
ApoE (n)	**non-E4**	104	3	36	16	< 0.0001	0.0015
	E4	64	16	27	40		
Age; years, Mean (SD)		70.1 (7.3)	73.2 (6.1)	71.7 (8.1)	71.9 (7.4)	0.0449	0.6547
Education; years, Mean (SD)		16.1 (2.6)	15.7 (2.4)	17.2 (2.2)	16.4 (2.7)	0.6462	0.0991
BMI; Mean (SD)		28.3 (5.7)	28.2 (4.9)	27.2 (3.7)	27.4 (5.7)	0.8915	0.3234
MMSE; Mean (SD)		28.6 (1.5)	28.0 (1.8)	28.2 (1.6)	26.7 (1.7)	0.132	< 0.0001

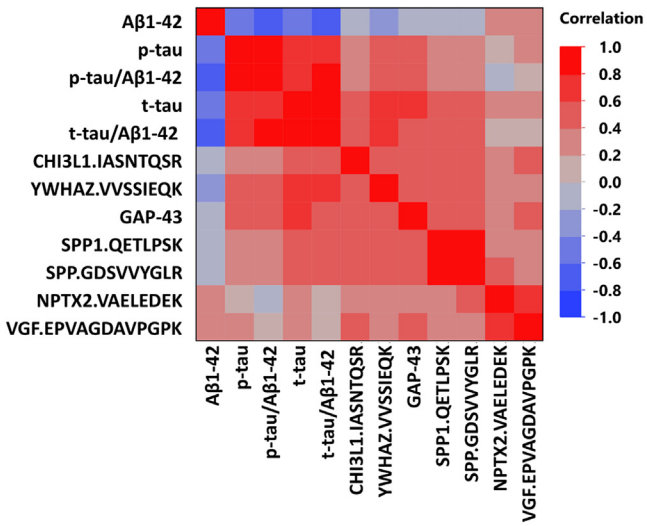

Fig. 2. Correlation heatmap of the A, T and N markers considered in this study, that are clustered based on their pairwise spearman correlation. As expected, the AT markers cluster closer together, followed by a group of N markers (CHI3L1, GAP-43, YWHAZ) with $\rho = 0.519$ to 0.631 pairwise correlation, the 2 peptide sequences of SPP1 with $\rho = 0.832$ correlation, and finally, NPTX2 and VGF clustered with $\rho = 0.741$ correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

scaling with severity of cognitive loss defining a diagnostic category.

Fig. 4 shows CSF levels of the 6 putative neurodegeneration markers examined in this study. As predicted from the correlation matrix shown in Fig. 2, CHI3L1, both species of SPP1, YWHAZ, and GAP-43 levels increased in the CSF with increased pathology, while both VGF and NPTX2 decreased. After adjusting for demographic factors, all markers varied significantly across the diagnostic groups ($p < 0.001$). In general, the differences across diagnostic categories among the putative neurodegeneration markers were less stark than those seen among AT markers (Fig. 3). However, what is not clear from Fig. 4 is whether these markers independently differentiate diagnostic categories or are redundant with AT markers, which will be addressed below.

3.4. CSF ATN markers as predictors of MCI to AD conversion

Fig. 5 illustrates that, as expected, low Aβ1–42 levels, high CSF t-tau or p-tau levels, or high ratios of either tau species to Aβ1-42, predict MCI to AD conversion over a 36-month period. These differences were significant for both EMCI and LMCI across all AT markers with $p < 0.001$. Fig. 6 shows baseline CSF levels of putative neurodegeneration markers in EMCI and LMCI subjects that either convert or do not convert to AD over a 36-month period. CSF CHI3L1, both species of SPP1, GAP-43 and YWHAZ levels were all higher in subjects that converted to AD over a 36-month period, and all were significant at $p < 0.05$ level. NPTX2 levels were significantly ($p < 0.05$) lower in converters, while VGF levels were not significantly different, but trended lower in converters. Further analysis of VGF levels after stratifying subjects using a previously published cutoff value for the ratio of t-tau to Aβ1-42 of 0.59 (Devanarayan et al., 2019) shows that in subjects with low ratios (i.e., normal AT markers), VGF levels are lower in MCI that converted to AD ($p = 0.0004$, Fig. 7A). Given the small number of subjects that converted from MCI to AD with a low t-tau/ Aβ1-42 ratio (n = 17), we have examined these relationships in an independent cohort, also from the ADNI dataset. Here, a different peptide fragment from VGF was measured (VGF_NSEPQDEGELFQGVDPR) and was found to have significantly lower levels in CSF of MCI to AD converters that had normal t-tau/ Aβ1-42 ratios (n = 20 converters, $p = 0.026$, Fig. 7B). Thus, across two independent cohorts, using two different fragments of VGF, these data suggest that VGF levels may be predictive of conversion to dementia in with negative AT biomarkers. Although these subjects did not have subsequent CSF analysis to determine if AT biomarkers converted to positive, 6 subjects in the first cohort had follow-up amyloid-PET scans. Four out of these 6 subjects had negative follow-up amyloid PET scans, suggesting that they may not have had AD despite a clinical diagnosis of AD. These data suggest that low CSF VGF may

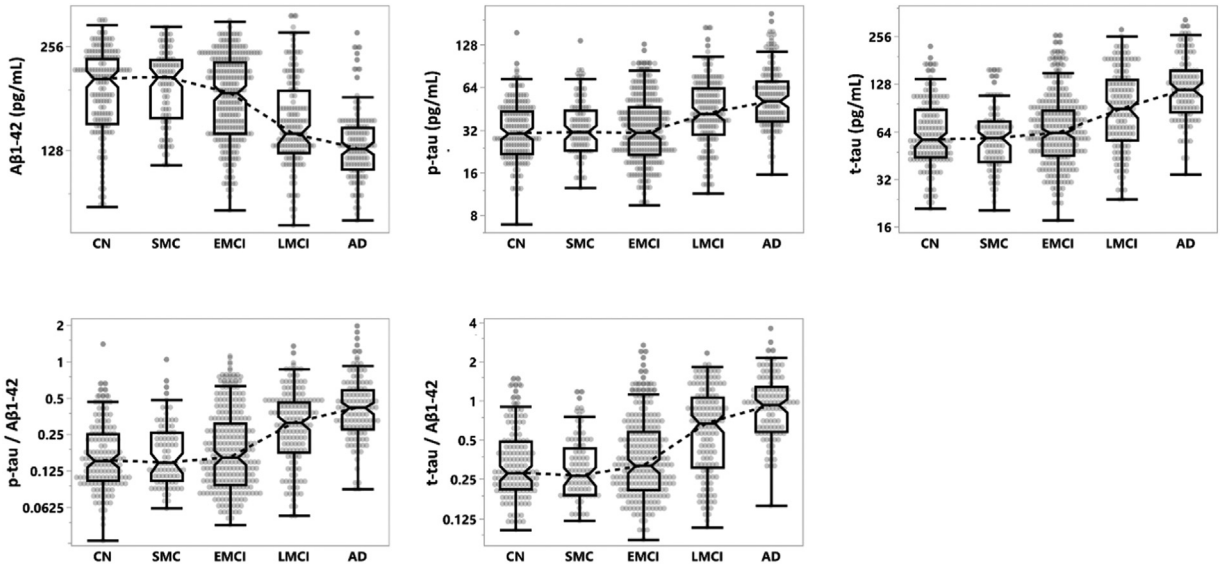

Fig. 3. Plot of the distribution of AT markers versus the diagnostic groups (age-matched normal control, CN; subjective memory complaints, SMC; early and late mild cognitive impairment, EMC and LMC; Alzheimer's disease, AD). Data are plotted in log2 scale as the markers are lognormally distributed. Because the markers are not significantly different between the CN and SMC groups, they are combined as a cognitively unimpaired (CUI) group. All markers are significantly different between the CUI and the other groups combined and also specifically versus AD ($p < 0.0001$), after adjusting for demographic features. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

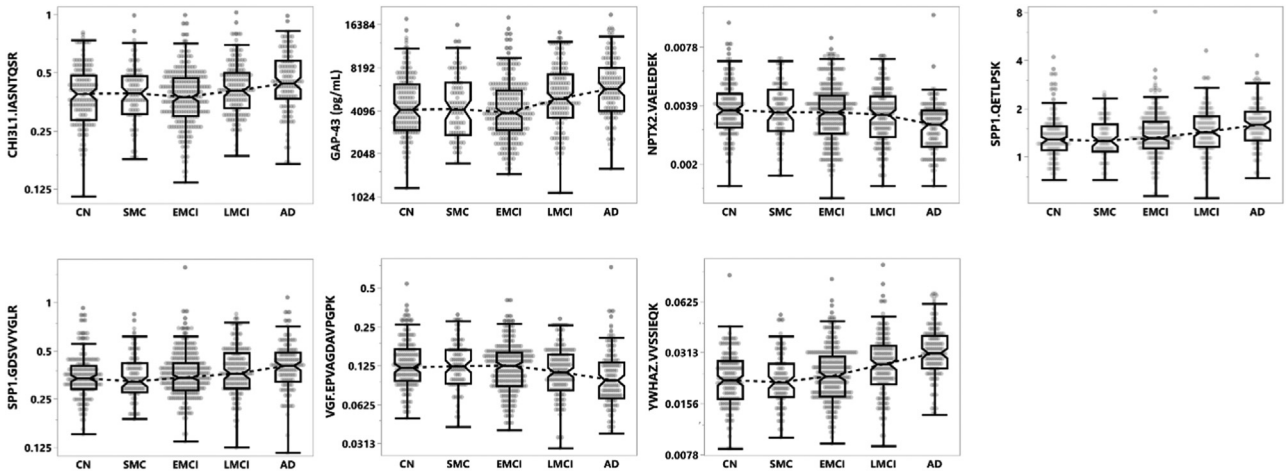

Fig. 4. Plot of the distribution of the 6 neurodegeneration (N) markers across different diagnostic groups. These markers are specific peptide sequence from each of 4 proteins (CHI3L1, NPTX2, VGF, YWHAZ), two peptide sequences from SPP1, and the full protein form of GAP-43. For all short peptides, data are reported as total area ratios, and are thus unitless. Data are plotted in log2 scale as the distributions are lognormally distributed. All markers are significantly different across the groups ($p < 0.001$), after adjusting for demographic features. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

be a general marker of neurodegenerative disease, as addressed in the Discussion. Future work using longitudinal sampling of CSF will determine if VGF changes do indeed precede AT changes in subjects that will develop clinical AD.

3.5. Combining CSF ATN markers as markers of disease state and markers to predict MCI to AD conversion

A+T+ putative N markers were compared in terms of their ability to differentiate CUI subjects from those with AD. Of the various ATN marker combinations, AT+VGF and AT+NPTX2 produced the highest balanced accuracy (BA, BA in each = 85.4%, See Table 3). We then used the markers optimized for differentiating disease state on an independent group of patients with either EMCI or LMCI to predict their conversion to AD over a 36-month period, allowing a head-to-head comparison of all A + T + N combinations (Table 3). The addition of VGF to AT markers significantly increased the prediction accuracy of conversion in EMCI subjects (from 66.5% to 76.5%, $p = 0.0012$), consistent with the findings in Fig. 7, which suggest that a low VGF level in CSF is an early predictor of cognitive decline. In the LMCI group, VGF, NPTX2, and YWHAZ, when combined with AT markers, predicted conversion to AD beyond the ability of AT markers alone, with VGF showing the greatest improvement (from 71.6% to 81.1%, $p = 0.0014$). Combining all biomarkers together (all species of amyloid, tau and all 6 putative biomarkers) irrespective of category did not improve the ability to predict MCI to AD conversion ($p > 0.05$).

Given the findings above that VGF, over a 3-year period, showed the greatest capacity in the ATN context to predict MCI to AD conversion, we examined the capacity of VGF, when combined with AT markers, to predict the time for MCI to AD conversion over a

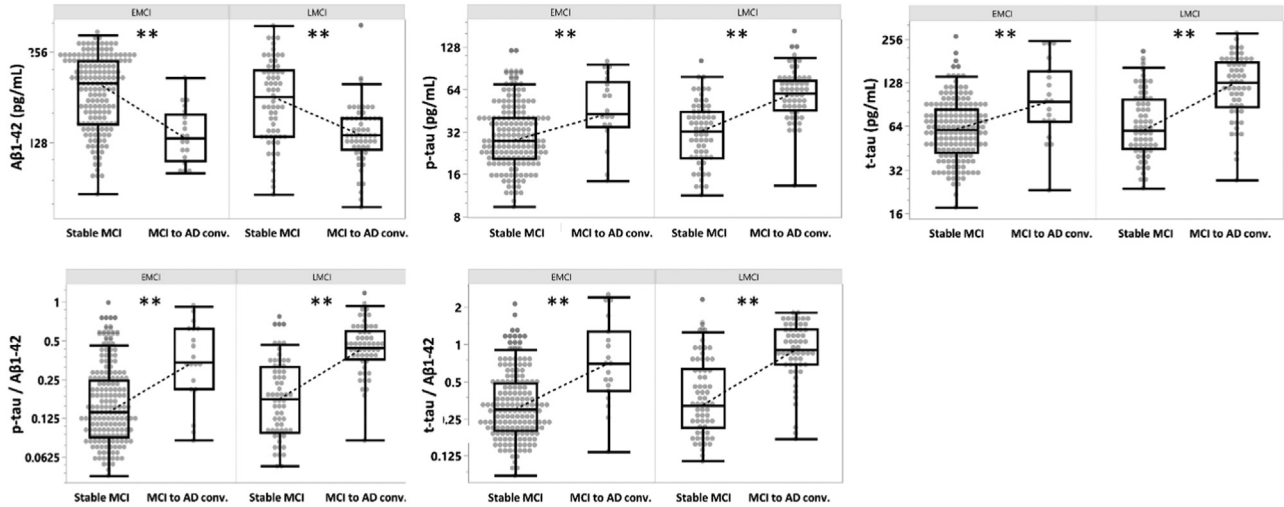

Fig. 5. Plot of the distribution of AT markers at baseline versus the 36-month future progression status of subjects that were diagnosed as either early or late mild cognitive impairment (EMCI, LMCI) at baseline. Data are plotted in log2 scale as the markers are lognormally distributed. All markers are significantly associated with the future progression of both EMCI and LMCI subjects (** denotes $p < 0.001$), after adjusting for demographic features. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

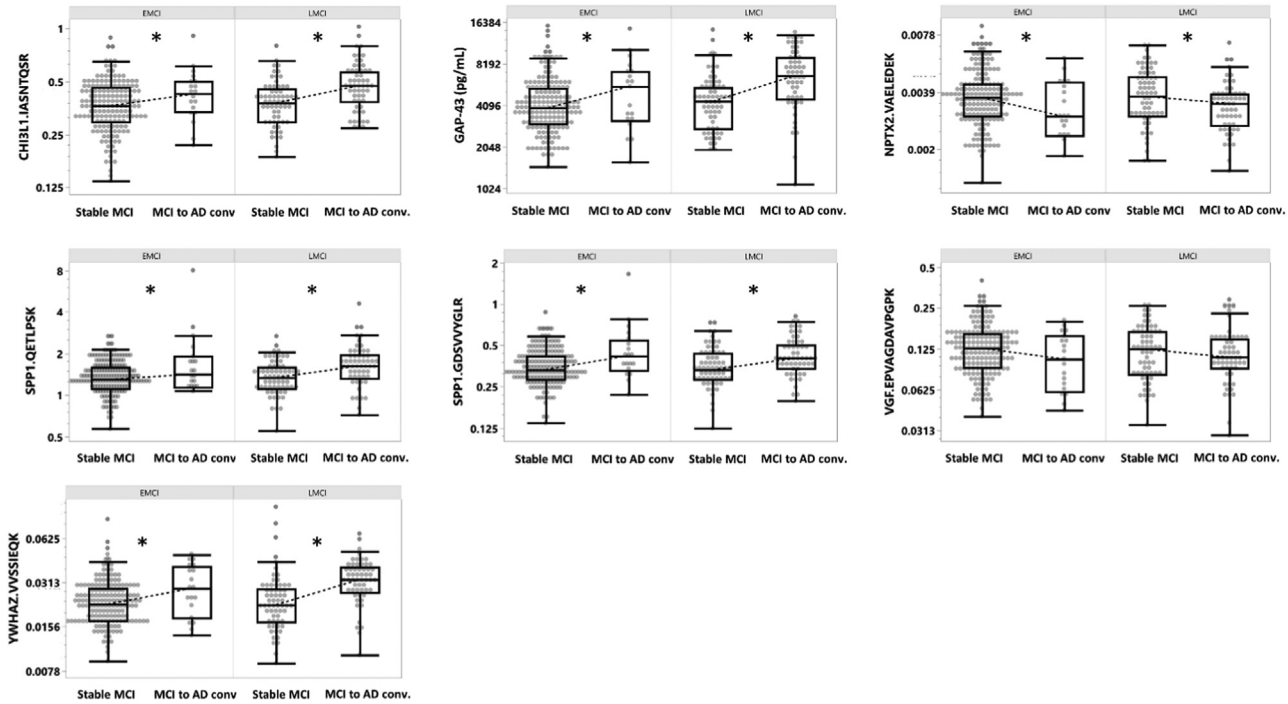

Fig. 6. Plot of the distribution of AT markers at baseline versus the 36-month future progression status of subjects that were diagnosed as either early or late mild cognitive impairment (EMCI, LMCI) at baseline. For all short peptides, data are reported as total area ratios, and are thus unitless. Data are plotted in log2 scale as the markers are lognormally distributed. All markers, except the VGF peptide, are significantly associated on their own with the future progression of either EMCI or LMCI subjects (* denotes $p < 0.05$), after adjusting for demographic features. However, as shown later in further analyses, VGF in combination with AT markers is significantly associated with MCI progression. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

10-year period using an analysis of non-progression over time. Using optimized markers for AT alone produced hazard ratios (HR) of 4.4 and 4.11 for EMCI to AD or LMCI to AD conversion, respectively (Fig. 8, top). Combining VGF with all AT markers increased the HRs to 6.7 and 9.88 for EMCI to AD ($p = 0.084$) or LMCI to AD conversion ($p = 0.0018$), respectively (Fig. 8, bottom). Consistent with the 3-year data shown in Table, 3, combining all biomarkers together did not improve the ability to predict MCI to AD conversion ($p > 0.05$).

Review of the relative influence of each feature within the A + T + VGF signature (initially defined to discriminate between AD and CUI) revealed that t-tau/Aβ42 ratio accounted for 52.3% of the influence with the next most important feature being VGF (21.5%, Fig. 9A). Analysis of the relationship between these two markers, indexed against the probability of MCI to AD conversion shows that: (1) the highest rate of conversion is seen when VGF levels are low and t-tau/Aβ1-42 levels are intermediate (yellow patch at bottom of the figure) and that (2) when t-tau/Aβ1-42 lev-

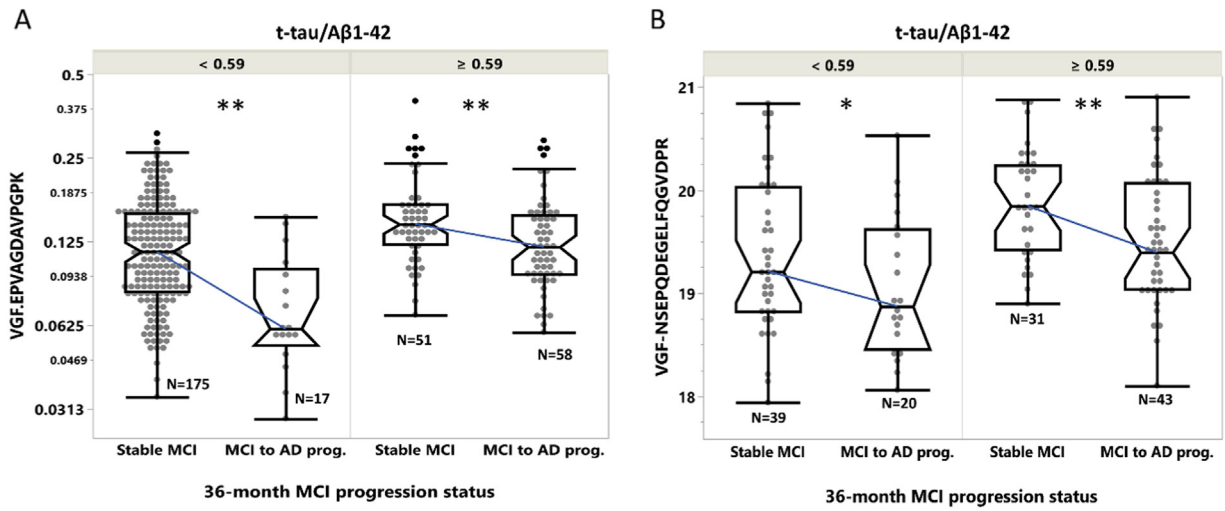

Fig. 7. (A) Association of VGF peptide with the progression of MCI subjects to AD is shown here for subjects with different levels of t-tau to Aβ1-42 ratio, especially in relation to a cut-point of 0.59 that has been reported as optimal for predicting MCI to AD progression (Llano et al., 2019). At t-tau to Aβ1-42 ratios lower than 0.59 (i.e., normal ratios), low levels were significantly associated with MCI to AD conversion (B) Identical analysis performed on a separate cohort from the ADNI database using a different peptide fragment from VGF (VGF_ NSEPQDEGELFQGVDPR, * denotes $p < 0.05$, ** denotes $p = 0.005$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

els are very high (rightmost portion of the plot), VGF levels are also relatively high and rates of MCI to AD conversion are low. The latter finding suggests that high levels of VGF in CSF may be a marker of neuroprotection to mitigate what would otherwise be strong indicator of MCI to AD progression.

4. Discussion

In the current study, we used machine-learning algorithms to derive optimal ATN signatures to distinguish AD from CUI individuals using combinations of CSF levels of Aβ1-42, t-tau, p-tau and their ratios, and peptides derived from a range of proteins involved in both synaptic function and inflammation. We examined the capacity for combinations of these signatures, using the ATN framework, to predict the conversion of an independent group of patients with MCI to develop AD over a 36-month and a 10-year period. We found that adding putative markers of neurodegeneration ("N" in the ATN framework) to Aβ and tau species ("AT") enhanced the prediction of conversion from MCI to AD by nearly two-fold. The N marker with the most predictive power, when combined with AT, was depressed levels of VGF in the CSF, which is a marker of synaptic function and plasticity. The implications of these results are discussed below.

4.1. Connection to previous work

Several CSF markers have been identified that, when coupled to changes in Aβ and tau or phosphorylated tau, enhance prediction of conversion from MCI to AD. For example, we reported that VGF-derived peptides, in combination with conventional biomarkers, significantly increased the ability to predict MCI to AD conversion (Llano et al., 2017; Llano et al., 2019). It is notable that the specific peptide fragments in marker in those cases were different than the 1 used in this study, and was done on an entirely different group of subjects. These findings support the notion that the findings regarding VGF are not related to a specific peptide in a particular subject pool, but to the parent protein (VGF) and are generalizable to the broader population. These findings are consistent with multiple other studies that observed declines in CSF VGF levels in AD subjects (Carrette et al., 2003; Hendrickson et al., 2015; Hölttä et al., 2014; Jahn et al., 2011; Selle et al., 2005). An additional peptide, neuronal protein tyrosine phosphatase receptor type N (PTPRN), may also have predictive power. For example, in a previous study from our group, a CSF peptide derived from PTPRN, when combined with AT markers and hippocampal volume, enhanced prediction of MCI to AD conversion (Devanarayan et al., 2019).

Other work using total tau as an N marker also showed that combining CSF AT markers with t-tau as a marker of neurodegeneration enhanced prediction of cognitive decline over time (Allegri et al., 2020; Delmotte et al., 2021). We should note that in the current study, t-tau was not used as a marker for neurodegeneration, but instead was incorporated into the tau ("T") category of markers. We did not explicitly test models where only p-tau was used as T and t-tau was used as N. However, because the signatures were entirely data-driven, our finding that AT+VGF outperformed AT alone demonstrates that using VGF as N outperforms any signature using t-tau as N because all combinations of AT markers were considered.

4.2. Biological implications of the markers examined in this study

We found that multiple peptides could differentiate between AD, MCI and control subjects (Fig. 4). The parent proteins from these peptides have been implicated in a host of functions related to synaptic physiology and plasticity as well as neural inflammation. For example, 2 markers of neuroinflammation: SPP1 and CHI3L1, were examined in this study. Osteopontin (OPN), referred to in the current study as SPP1 (secreted phosphoprotein 1), is an extracellular phosphoprotein expressed in response to stress and injury (Chai et al., 2021; Frigerio et al., 2019). There is an emerging literature implicating brain inflammation with AD. For example, recent work has reported co-localization of activated microglia and astrocytes with amyloid plaques in AD (Arends et al., 2000), together with elevated levels of pro-inflammatory cytokines in CSF (Llano et al., 2012). In addition, several studies have revealed that brain and/or CSF samples obtained from AD patients contain higher OPN levels than controls (Chai et al., 2021; Comi et al., 2010; Sun et al., 2013). An additional marker of neural inflamma-

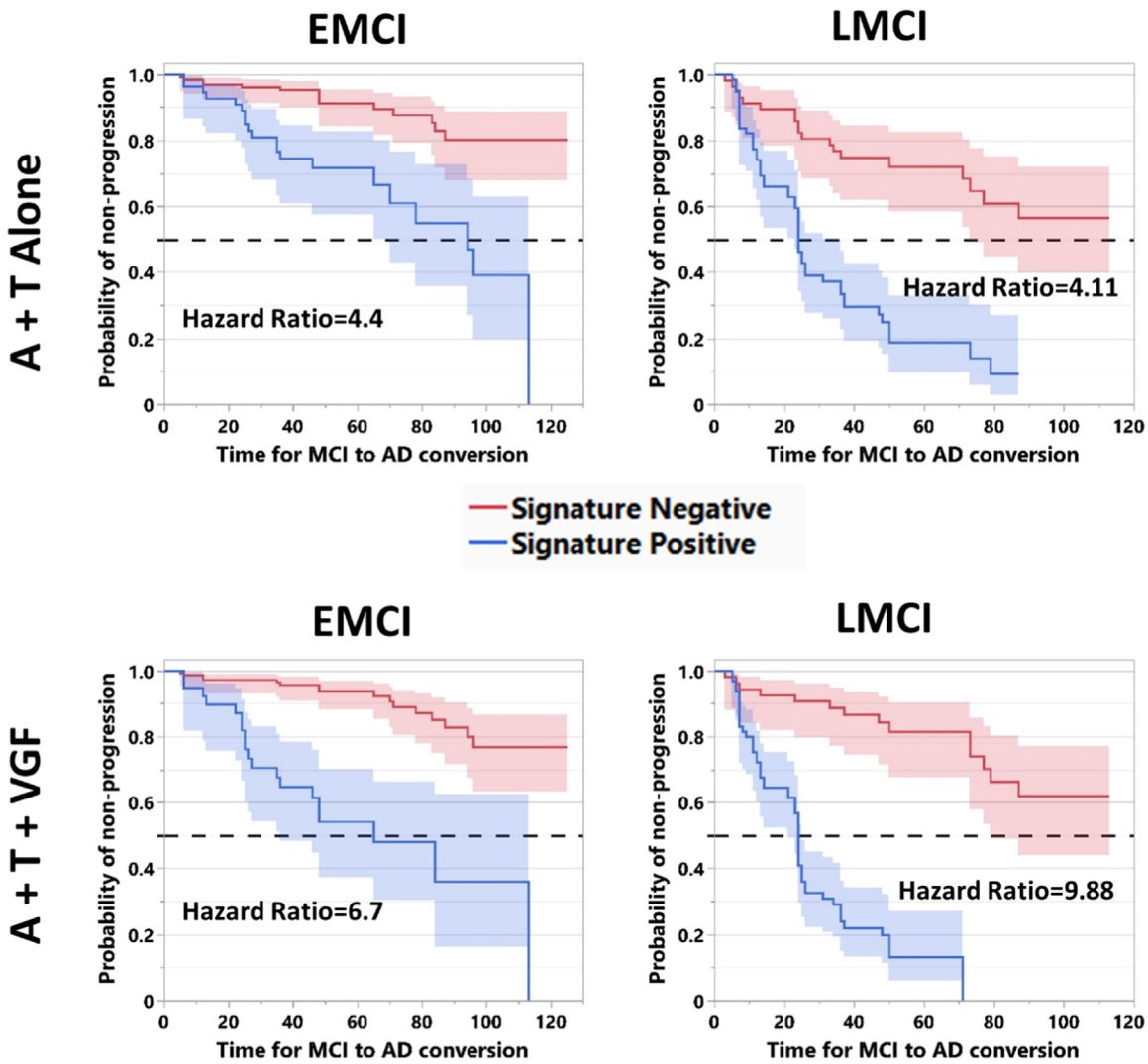

Fig. 8. Top row shows the Kaplan-Meier plots of the time to progression from MCI to AD which reveal considerably faster progression of the EMCI and LMCI subjects ("signature positive") that were predicted at baseline as faster progressors to AD during the 10-year clinical follow-up using AT only markers. Bottom row shows similar plots for AT markers + VGF. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

tion examined in this study is Chitinase 3-like 1 (CHI3L1). CHI3L1 is a secreted glycoprotein, and its parent gene is expressed in microglia and astrocytes in association with neuroinflammation (Sanfilippo et al., 2019). Its role remains unknown, but several studies have found an association of CHI3L1 with several inflammatory diseases, and it has been found to be a marker of neuroinflammation in AD (Moreno-Rodriguez et al., 2020; Sanfilippo et al., 2019). CHI3L1 expression is found in astrocytes situated near blood vessels as well as the neuropathological hallmarks of AD: Aβ plaques, and neurofibrillary tangles (Bonneh-Barkay et al., 2010). Recent work also showed that CHI3L1-positive astrocytes were significantly increased in frontal cortex and white matter in severe AD (Moreno-Rodriguez et al., 2020) and that elevated levels are present in the CSF of AD subjects (Zhang et al., 2018).

In addition, the levels of several markers of synaptic function were found in the current study to be altered in AD and predictive of MCI to AD conversion. The parent protein for YWHAZ is a 14-3-3 protein encoded by the YWHA family of genes (YWHAE, YWHAZ, and YWHAQ). 14-3-3 zeta protein isoform 1 (YWHAZ) is a regulatory protein that is associated with tau phosphorylation in neurofibrillary tangles in AD (Kim et al., 2015; Yang et al., 2020). YWHAZ protein expression is reduced in the hippocampus of AD patients (Kim et al., 2015) and increased levels have been reported in the CSF of AD patients (Bader et al., 2020; Zhou et al., 2020). CSF GAP-43 levels were also found in the current study to be elevated in the CSF of AD patients and were predictive of MCI to AD conversion. GAP-43 is a synaptic protein found in presynaptic terminals and important for neuronal development and synaptogenesis (Sandelius et al., 2019). Thus, the current study extends and confirms previous work that reported GAP-43 to be elevated in CSF in the presence of AD pathology (Milà-Alomà et al., 2021; Remnestål et al., 2016; Sandelius et al., 2019; Tible et al., 2020).

NPTX2 was found to independently contribute to prediction of MCI to AD conversion when combined with AT, suggesting that it may serve as a neurodegeneration marker in the ATN framework. NPTX2 is a secreted glycoprotein and is likely involved in AMPA-mediated excitatory synapse assembly. Regulation of the trafficking of AMPA receptors is essential for synaptic plasticity, and NPTX2 has been associated with AMPA-mediated excitatory synaptogenesis (Chapman et al., 2020; Libiger et al., 2021). Previous work reported decreases NPTX2 in brain and CSF of MCI

Table 3
Performance of the ATN signatures for disease diagnosis and prognostic prediction of disease progression. N.all = all putative neurodegeneration markers

Scenarios	Internal Cross-Validation (CUI vs. AD)			Test Set (Early MCI to AD progression in 36m)					Test Set (Late MCI to AD progression in 36m)				
	Sens.	Spec.	BA	Sens.	Spec.	BA	ROC AUC	p-val vs. AT	Sens.	Spec.	BA	ROC AUC	p-val vs. AT
AT	*84.0%*	*81.4%*	82.7%	52.6%	80.4%	**66.5%**	78.8%		75.0%	68.3%	**71.6%**	77.2%	
N.all	*81.1%*	*86.0%*	***83.6%***	70.0%	76.0%	**73.0%**	79.8%	0.4279	77.6%	81.0%	**79.3%**	86.9%	*0.0267*
AT + N.all	*84.0%*	*86.4%*	***85.2%***	73.7%	82.7%	**78.2%**	85.1%	*0.0702*	87.5%	79.4%	**83.4%**	89.3%	*< 0.0001*
AT + VGF	*82.1%*	*88.6%*	***85.4%***	68.4%	84.5%	**76.5%**	87.7%	***0.0012***	87.5%	74.6%	**81.1%**	84.9%	*0.0014*
AT + NPTX2	*84.0%*	*86.8%*	***85.4%***	73.7%	82.1%	**77.9%**	83.5%	*0.0535*	78.6%	76.2%	**77.4%**	86.4%	*0.0003*
AT + GAP43	*83.0%*	*80.5%*	***81.7%***	63.2%	76.8%	**70.0%**	77.3%	*0.8615*	85.7%	71.4%	**78.6%**	79.8%	*0.0816*
AT + SPP	*84.9%*	*80.9%*	***82.9%***	57.9%	79.8%	**68.8%**	77.2%	*0.8791*	82.1%	69.8%	**76.0%**	79.4%	*0.0667*
AT + YWHAZ	*83.0%*	*81.8%*	***82.4%***	52.6%	78.6%	**65.6%**	76.8%	*0.8865*	78.6%	68.3%	**73.4%**	80.3%	*0.0126*
AT + CHI3L1	*84.9%*	*80.0%*	***82.5%***	57.9%	78.6%	**68.2%**	74.5%	0.9415	71.4%	66.7%	**69.0%**	78.6%	0.1619

and AD subjects (Libiger et al., 2021; Xiao et al., 2017), and that CSF levels can predict cognitive decline (Galasko et al., 2019; Swanson et al., 2016). Previous work has also shown that CSF levels decrease more rapidly in progressors than nonprogressors and these declines also correlated with declines in cognitive function, as measured using the MMSE or ADAS-Cog13 (Libiger et al., 2021).

4.3. VGF levels in the ATN framework predict MCI to AD conversion

Of the peptides examined in the current study, low VGF levels in the CSF were found to most strongly predict MCI to AD conversion in the ATN framework. In addition, low VGF levels in the CSF were predictive of MCI conversion, even in the presence of a normal ratio of t-tau to Aβ42, as shown using 2 independent cohorts from the ADNI database and 2 different peptide fragments from VGF (Fig. 7). The finding that a minority of these subjects with negative AT biomarkers that converted to clinical AD developed positive amyloid PET scans suggests that these subjects may not have had amyloid deposition, consistent with the finding that low CSF VGF can be found in non-AD dementia (Rüetschi et al., 2005; van Steenoven et al., 2019). In addition, the relationship between VGF and MCI to AD conversion is not straightforward, as evidenced by the fact that univariate analysis of VGF levels in MCI subjects was not predictive of AD conversion (Fig. 7). In contrast, when used in conjunction with AT markers, VGF becomes highly predictive (Fig. 8). These data likely derive from the complex relationship between these markers and AD conversion shown in Fig. 9B. It is clear that low VGF levels on their own are not predictive of conversion (lower left of 9B). However, low levels of VGF in the presence of abnormal t-tau/Aβ42 ratios is highly predictive, consistent with the Kaplan-Meier plots shown in Fig. 8. These data point to the need to perform multivariate analyses of multiple markers, rather than relying on individual markers to predict MCI to AD conversion.

VGF is a widely distributed neuronal protein (Alder et al., 2003; Cocco et al., 2010; Snyder and Salton, 1998) whose levels are upregulated by neuronal activity (Snyder et al., 1997). VGF is important for expression of synaptic plasticity and neurogenesis, as shown in animal models (Alder et al., 2003; Bozdagi et al., 2008; Thakker-Varia et al., 2014; Thakker-Varia et al., 2007). Overexpression of VGF can protect against AD pathology, which is consistent with the current data suggesting that its levels are lowered in AD subjects and MCI subjects that will later convert to AD (Fig. 7) and that high levels may mitigate the impact of having strongly positive AT markers (Fig. 9B). Multiple recent studies have implicated VGF in the pathogenesis of AD (Beckmann et al., 2020; El Gaamouch et al., 2020; Llano et al., 2017; Llano et al., 2019), reviewed in (Quinn et al., 2021). It is unlikely that the drop in VGF levels in the CSF in the current study represents a shift to parenchymal accumulation, because low levels are seen in the cerebral cortex of AD subjects (Cocco et al., 2010) and because low VGF CSF levels are seen in other neurodegenerative diseases such as frontotemporal dementia and dementia with Lewy bodies (Rüetschi et al., 2005; van Steenoven et al., 2019), suggesting that low CSF levels are a sign of neuronal loss. Given that depressed CSF levels are seen in other neuropathological states besides AD, it is possible that the low VGF levels in this study may have been related to other disease states, consistent with the variable specificity for AD found with clinical diagnosis (Beach et al., 2012; Franklin et al., 2015; Kim et al., 2020; Sabbagh et al., 2017).

It is worth noting that although the ATN framework provides an intuitive method for grouping putative biomarkers, not all biomarkers easily fit into these 3 bins. For example, markers of inflammation or disrupted metabolism do not involve pri-

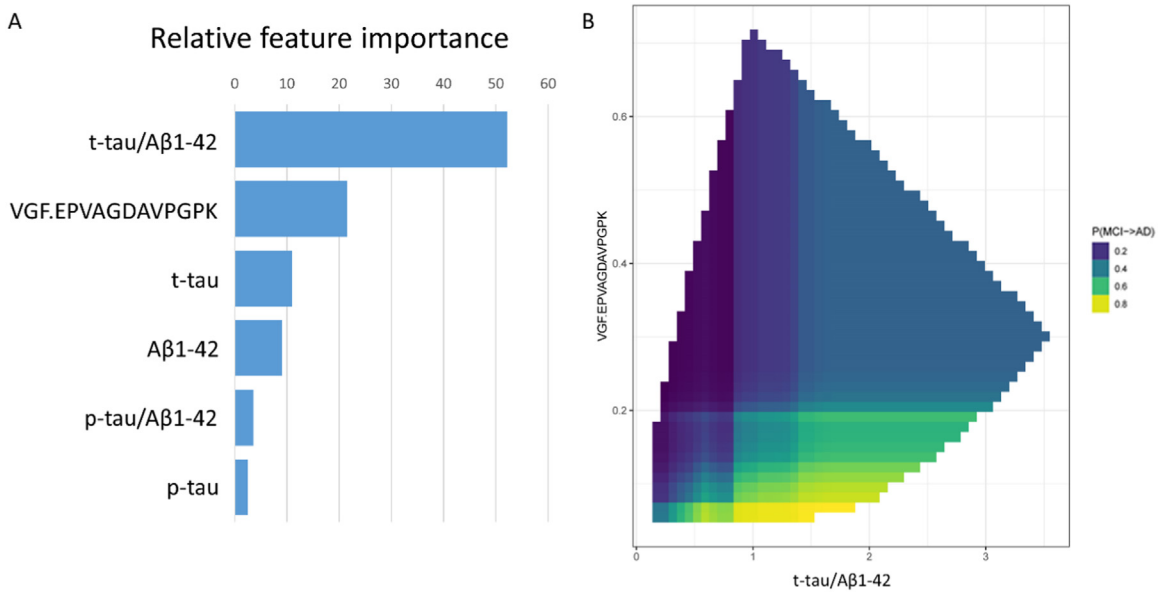

Fig. 9. (A) The relative influence plot of the predictors in this signature reveals that VGF is a valuable contributor to the prediction when combined with the ratio of t-tau to Aβ1-42. (B) The interaction prediction profile, where the color gradient from blue to green to yellow represents increasing likelihood of progression from MCI to AD, reveals a strong dependence between VGF and the ratio of t-tau to Aβ1-42, with the lower levels of VGF along with higher ratio of t-tau to Aβ1-42 at baseline resulting in greater likelihood of future progression from MCI to AD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mary pathology of amyloid, tau, nor are they a direct reflection of neurodegeneration. For this reason, other frameworks, such as the ATNX framework have been proposed whereby "X" represents other pathologies not encompassed by A, T, or N (Huang et al., 2022). In the current study, in addition to examining biomarkers within the A, T or putative N groups, we also examined the performance of all biomarkers (all species of amyloid beta, tau and all 6 novel markers), regardless of category (Table 3). We found that the performance of the combined biomarker (which does not rely on ATN groupings) did not differ in any substantive way compared to A + T + VGF. These data suggest that an additional "X" category, chosen from the examined biomarkers, would not have improved biomarker performance. This finding does not exclude the possibility that other, unmeasured, biomarkers could be combined with A + T+ VGF to improve overall performance in predicting MCI to AD conversion.

5. Conclusion

The current study provides additional evidence that multiple candidate peptides may serve as markers of neurodegeneration in the CSF and, when combined with AT markers, VGF most strongly enhances the ability to predict whether MCI subjects convert to AD. As such, VGF may serve as an N marker in the ATN framework and may be useful clinically to predict which MCI patients are most likely to convert to AD, and thus most likely to benefit from aggressive intervention, such as amyloid-lowering therapy. The use of VGF + AT markers in CSF has the additional advantage of all being obtained as a single patient encounter, without the need to incorporate expensive imaging testing, which may not be available in remote areas. In addition, use of CSF AT+VGF levels in clinical trials may help to select subjects with the fastest rate of cognitive decline and thus may be used to accelerate the development of new therapeutic interventions by shortening clinical trial time. Future work will help to clarify important questions, such as the specificity of these markers for AD compared to other neurogenerative disorders, and whether these markers may themselves have therapeutic implications (e.g., elevating brain levels of VGF as a therapeutic intervention).

Credit author statement

VD helped conceive the study, performed all analyses and contributed to figure development.

PD helped to write the manuscript.

DL helped conceive the study, contributed to figure development and helped to write the manuscript.

Disclosure statement

DAL has consulted for Eisai Inc., Boston HealthCare and Techspert.io in the past year, VD is an employee of Eisai, Inc.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2022.07.015.

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