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RESEARCH ARTICLE

Cerebrospinal fluid biomarker profiling of diverse pathophysiological domains in Alzheimer's disease

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Abstract

INTRODUCTION: While Alzheimer's disease (AD) is defined by amyloid-β plaques and tau tangles in the brain, it is evident that many other pathophysiological processes such as inflammation, neurovascular dysfunction, oxidative stress, and metabolic derangements also contribute to the disease process and that varying contributions of these pathways may reflect the heterogeneity of AD. Here, we used a previously validated panel of cerebrospinal fluid (CSF) biomarkers to explore the degree to which different pathophysiological domains are dysregulated in AD and how they relate to each other.

METHODS: Twenty-five CSF biomarkers were analyzed in individuals with a clinical diagnosis of AD verified by positive CSF AD biomarkers (AD, $n = 54$) and cognitively unimpaired controls negative for CSF AD biomarkers (CU-N, $n = 26$) using commercial single- and multi-plex immunoassays.

RESULTS: We noted that while AD was associated with increased levels of only three biomarkers (MMP-10, FABP3, and 8OHdG) on a group level, half of all AD participants had increased levels of biomarkers belonging to at least two pathophysiological domains reflecting the diversity in AD. LASSO modeling showed that a panel of FABP3, 24OHC, MMP-10, MMP-2, and 8OHdG constituted the most relevant and minimally correlated set of variables differentiating AD from CU-N. Interestingly, factor analysis showed that two markers of metabolism and oxidative stress (24OHC and 8OHdG) contributed independent information separate from MMP-10 and FABP3 suggestive of two independent pathophysiological pathways in AD, one reflecting neurodegeneration and vascular pathology, and the other associated with metabolism and oxidative stress.

DISCUSSION: Better understanding of the heterogeneity among individuals with AD and the different contributions of pathophysiological processes besides amyloid-β and tau will be crucial for optimizing personalized treatment strategies.

KEYWORDS
Alzheimer's disease, biomarkers, cerebrospinal fluid, FABP3, 8OHdG, heterogeneity, metabolic, MMP10, pathophysiological processes, profiling, vascular

Highlights

- A panel of 25 highly validated biomarker assays were measured in CSF.
- MMP10, FABP3, and 8OHdG were increased in AD in univariate analysis.
- Many individuals with AD had increased levels of more than one biomarker.
- Markers of metabolism and oxidative stress contributed to an AD multianalyte profile.
- Assessing multiple biomarker domains is important to understand disease heterogeneity.

1 | BACKGROUND

Molecular biomarkers have come to play important roles in the diagnosis of Alzheimer's disease (AD). Amyloid-β PET imaging and cerebrospinal fluid (CSF) levels of amyloid-$\beta_{x\text{-}42}$ (Aβ_{42}), total tau (tTau), and phospho-tau (pTau) are now considered to be almost as good as autopsy for the diagnosis of AD.[1] These biomarkers are strongly associated with the presence of amyloid-β plaques and/or paired helical filament tau neurofibrillary tangles in the brain that are the signature neuropathological lesions of AD.[2] It is, however, evident that many additional pathophysiological processes such as inflammation,[3,4] neurovascular dysfunction,[5] oxidative injury,[6,7] and other metabolic derangements[8] also contribute to the neurodegenerative process. These mechanisms act in pathophysiological cycles as the disease progresses,[9,10] eventually resulting in synaptic loss through microglial phagocytosis and impaired repair plasticity, and ultimately causing neuron death through apoptosis[11] and necrosis.[12–14] This complexity in the disease process combined with the high prevalence of co-morbid pathologies[15] renders it impossible to fully characterize AD with a single biomarker. Broadening the array of biomarkers beyond amyloid and tau is important to characterize the degree to which the different associated processes of inflammation, vascular injury, metabolic derangements, and others may contribute at the disease and at the individual levels. We hypothesize that varying contributions of these processes reflect the heterogeneity within AD in terms of risk factors and genetics, regional pathological vulnerability, co-morbid illness and pathologies, symptom profiles, progression, and likely treatment responses. Understanding this heterogeneity is necessary to guide personalized treatment strategies and choose appropriate combinations of treatments for different people at different phases of disease.

We previously reported a fit-for-purpose validation approach to qualify a broad selection of commercially available immunoassays across multiple pathophysiological domains for use in CSF samples in clinical trials.[16] Beyond amyloid-β and tau, we identified high-performing assays spanning neurodegeneration/neural injury, inflammation, vascular injury, and metabolism. Here, we applied a focused panel of assays of 25 analytes to describe multi-pathophysiological profiles of AD in comparison to cognitively unimpaired controls.

2 | METHODS

2.1 | Study participants

A "high-contrast" cohort consisting of 80 CSF samples was selected from the MassGeneral Institute for Neurodegenerative Disease (MIND) Tissue Bank for Biomarker Discovery (n = 30) and the Penn Memory Center biorepository (PMC, n = 50). Clinical diagnoses were verified in consensus conference and/or chart review by an experienced neurologist (S.E.A.) according to the 2011 National Institute of Aging—Alzheimer's Association diagnostic criteria for AD[17] and MCI due to AD.[17,18] AD status was furthermore verified by CSF AD biomarker status[1] (A$\beta_{42/40}$ ratio, pTau181, and tTau using in-house derived thresholds for AD [Figure S1]) measured by Euroimmun enzyme-linked immunosorbent assay (ELISA) assays (Lübeck, Germany). The cohort consisted of two groups (Table 1):

(1) AD (n = 54) consisting of AD dementia (n = 39) and mild cognitive impairment due to AD (MCI; n = 15). Mini-Mental State Examination (MMSE) scores were available in 42 participants (median: 23.5; interquartile range: 21.7–26).

(2) Cognitively unimpaired participants negative for AD biomarkers (CU-N; n = 26), consisting of healthy volunteers (HC; n = 17) and individuals with other non-dementia-causing neurological disorders (OND; n = 9).

2.2 | Sample collection and biomarker analysis

Selected CSF samples were collected by lumbar puncture with adherence to ADNI CSF collection protocols,[19] including rapid processing and freezing using low-binding polypropylene collection tubes and cryovials, and stored at −80°C until use.

TABLE 1 Demographic and AD biomarker information.

	AD ($n = 54$)	CU-N[a] ($n = 26$)	p-Value
Age, years			
Median (range)	71 (49–86)	69 (48–83)	0.10
Sex, *n* (%)			
Female	31 (57%)	11 (42%)	0.3
Male	23 (43%)	15 (58%)	
$A\beta_{42/40}$ ratio			
Mean (SD)	0.038 (0.014)	0.113 (0.026)	<0.001
tTau, pg/mL			
Mean (SD)	940 (269)	556 (218)	<0.001
pTau-181, pg/mL			
Mean (SD)	142.1 (36.9)	43.4 (31.2)	<0.001
APOE e4, *n* (%)			
e4	31 (57%)	5 (19%)	<0.002
No e4	11 (20%)	15 (58%)	
Unknown	12 (22%)	6 (23%)	

[a]Cognitively unimpaired controls (CU-N) consisting of healthy volunteers ($n = 17$) and individuals with other non-dementia-causing neurological disorders [neuropathies ($n = 4$), headaches ($n = 1$), idiopathic intracranial hypertension ($n = 1$), essential tremor ($n = 1$), stiff-person syndrome ($n = 1$), and spinocerebellar atrophy ($n = 1$)]. Adjusted p-values (Benjamini-Hochberg) were calculated using unpaired t-tests for age, $A\beta_{42/40}$ ratio, tTau, and pTau181. Fisher's exact test was used for sex and *APOE* e4 carrier status.

Abbreviations: AD, Alzheimer's disease; *APOE* e4, apolipoprotein *E* e4; SD, standard deviation.

Biomarker levels were measured using commercial single- and multi-plex immunoassays that were previously validated in-house for sensitivity and technical reproducibility in CSF.[16] Twenty-six biomarkers displaying high precision and short-term longitudinal stability in healthy volunteers were validated for the CSF biomarker panel, of which 25 passed the initial QC analysis (Table 2). All assays were performed in duplicate according to manufacturer protocols. Median coefficient of variation (CV) for replicates for the different analytes was 6.1 ± 11.0% (mean ± SD) and all analytes except vascular endothelial growth factor-C (VEGF-C), which was excluded from subsequent analyses, had a median CV <15% (Table S1). Samples were randomized over multiple plates and assay performance and QC analysis were performed blinded to the clinical groups. Four pooled QC samples were included on all plates to normalize for plate-to-plate variability. Normalization was performed by dividing analyte concentrations by the normalization factor for the plate the sample was measured on. Normalization factors were derived from the ratio of the individual plate QC concentrations to the mean QC concentration across all plates. Median plate-to-plate CVs for the QC samples was 14.6 ± 9.0% (mean ± SD) before normalization and 8.2 ± 6.0% after normalization (Table S1).

Apolipoprotein E (APOE) e4 carrier status was determined using a colorimetric ELISA (BioVision, Milpitas, CA), which had been previously validated in-house for specificity and sensitivity for *APOE* e4 phenotype in CSF from individuals with known genotype.

RESEARCH IN CONTEXT

1. **Systematic review**: The authors reviewed existing literature on cerebrospinal fluid biomarkers and pathophysiological processes in Alzheimer's disease (AD) using PubMed, Google Scholar, and reference lists from relevant papers. These relevant citations are appropriately cited.
2. **Interpretation**: Our findings indicated a heterogeneity in biomarker profiles on an individual level and suggested that biomarkers for metabolism and oxidative stress contributed independent information to more established biomarker for neurodegeneration consistent with existing literature showing that AD is a complex disease that cannot be fully explained by a single biomarker.
3. **Future directions**: The development of assays profiling the contributions of the different pathophysiological processes associated with AD is needed to understand the heterogeneity of the disease process and to guide personalized treatment strategies.

2.3 | Statistical analysis

Statistical analyses were completed in R (4.0.2 GUI 1.72 Catalina build) and RStudio (Version 1.3.1056). All biomarker data was log transformed prior to statistical analysis due to non-normal distribution. Between-group differences (AD vs. CU-N) of key demographic variables were analyzed via independent sample *t*-tests for continuous variables and with the Fisher's exact test for categorical variables.

To investigate the effects of demographic variables (age, sex, and *APOE* e4 carrier status) and AD biomarkers ($A\beta_{42/40}$ ratio, tTau, and pTau-181) on each analyte, standard linear regression models (analyte ~ covariate) were performed. The regression models were run separately by diagnosis (AD vs. CU-N) to examine the influence of these covariates on analyte within each sub-group. Multivariate models were run for each analyte to examine the association with diagnostic group, using a standard linear regression model (analyte ~ diagnosis + age + sex) using all samples ($n = 80$) and separately in a sensitivity analysis using only AD and HC samples ($n = 71$). All p-values were adjusted using the Benjamini-Hochberg method, and significance set at $p < 0.05$.

To assess heterogeneity of biomarker profiles, individuals were classified as positive for a biomarker domain if they had at least one biomarker in that domain that exceeded 2 SD of the mean of the CU-N group. Fisher's exact test was used to compare frequency of positive markers across groups (AD vs. CU-N).

Age and log-transformed biomarker concentrations were *z*-score transformed and standardized to 0–1 for the following analysis. The least absolute shrinkage and selection operator (LASSO) logistic

TABLE 2 Assays included in the cerebrospinal fluid multi-pathophysiology panel (CMP3).

	Vendor	Assay	Catalog number	Dilution factor
AD biomarkers				
Aβ42	Euroimmun	Beta-amyloid (1–42) Assay	EQ 6521-9601-L	1:1
Aβ40	Euroimmun	Beta-amyloid (1–40) Assay	EQ 6511-9601-L	1:21
Total tau	Euroimmun	Total tau assay	EQ 6531-9601-L	1:1
Phospho-tau 181 (pTau181)	Euroimmun	P-Tau (pT181) Assay	EQ 6591-9601-L	1:1
Neurodegeneration				
NfL	Quanterix	NF-light Simoa assay advantage kit	103186	1:25
FABP3	MSD	Human FABP3 kit	K151HTD	1:2
YKL-40	MSD	Human YKL-40 kit	K151NHD	1:50
Inflammation				
IL-6, IL-7, IL-8, IL-12/IL-23p40, IL-15, IL-16, MCP-1, MDC, MIP-1β	MSD	Special order human biomarker assay	N05JA	1:2
Vascular injury				
Flt-1, PlGF, VEGF, VEGF-C[a], VEGF-D	MSD	V-PLEX angiogenesis panel 1	K15190D	1:2
ICAM-1, VCAM-1	MSD	V-PLEX Vascular injury panel 2	K15198D	1:5
MMP-2, MMP-10	MSD	Human MMP 2-plex ultra-sensitive panel	K15033C	1:2
Metabolism				
8-OHdG	Cell biolabs, Inc.	OxiSelect oxidative DNA damage ELISA	STA-320	1:2
24-OHC	Enzo lifesciences	24(S) hydroxycholesterol ELISA	ADI-900-210-0001	1:2
Adiponectin	MSD	Human adiponectin kit	K151BXC	1:10
Leptin	MSD	V-PLEX human leptin kit	K151V5D	1:2
Soluble IR	BioVendor	Insulin receptor human ELISA	RD1991041200R	1:2

[a]VEGF-C did not meet quality control criteria and was not included in further analysis. Analytes were grouped into five pathophysiological domains based on their predominant biological activities: AD biomarkers, neurodegeneration, inflammation, vascular injury, and metabolism.
Abbreviation: AD, Alzheimer's disease.

regression was performed to select a panel of predominant biomarkers that distinguish AD from CU-N. Resampling techniques were used to understand the confidence of the LASSO model: An 80%/20% random split on the entire dataset was performed 1000 times to create 1000 unique training and test sets. These training and test sets had an equal proportion of CU-N (34%) and AD (66%) participants to simulate the proportion of AD participants in the entire dataset. LASSO logistic regression was performed on each of the 1000 training sets. In each LASSO model, a five-fold cross-validation method was used to determine the best penalty for minimizing error. Python and R "glmnet" and "pROC" packages were used for analyses.

To assess the importance of each biomarker, two metrics were used: (1) the number of times the biomarker remained in the 1000 LASSO models, and (2) the coefficients of each biomarker. Next, principal component analysis (PCA) was performed to categorize biomarkers based on the eigenvectors. The variance of each biomarker was standardized across all the dimensions and plotted via a heatmap. The heatmap.2() function in the R gplots package was used to generate a heatmap of the Pearson correlation coefficient matrix, combined with a dendrogram to visualize the degree of collinearity between analytes.

3 | RESULTS

3.1 | Sample characteristics and demographic effects

Participant characteristics are presented in Table 1. There were no differences in age nor sex between the AD and the CU-N subgroups. The AD subgroup contained a greater proportion of *APOE* e4 carriers (57% vs. 19%; $p < 0.002$) as expected.[20] Initial analysis showed that 8 biomarkers increased with age within the CU-N sub-group (Figure S2). The correlation was moderately strong with an $r^2 > 0.4$ for IL-15 ($r^2 = 0.49$) and YKL-40 ($r^2 = 0.42$), while the correlation was weak for the remaining markers (ICAM1: $r^2 = 0.30$; VCAM-1: $r^2 = 0.29$; MCP-1: $r^2 = 0.26$; MMP2: $r^2 = 0.26$; PlGF: $r^2 = 0.24$; and FABP3: $r^2 = 0.19$). There were no effects of sex on any of the biomarkers in the CU-N subgroup. All subsequent analyses were performed with age and sex as co-variates unless specified otherwise. CSF levels of FABP3 ($p < 0.01$), MMP-2 ($p < 0.01$), NfL ($p < 0.01$), VEGF ($p < 0.05$), and YKL-40 ($p < 0.001$) were increased in individuals with AD carrying at least one *APOE* e4 allele compared to non-carriers (Figure S3), while the small

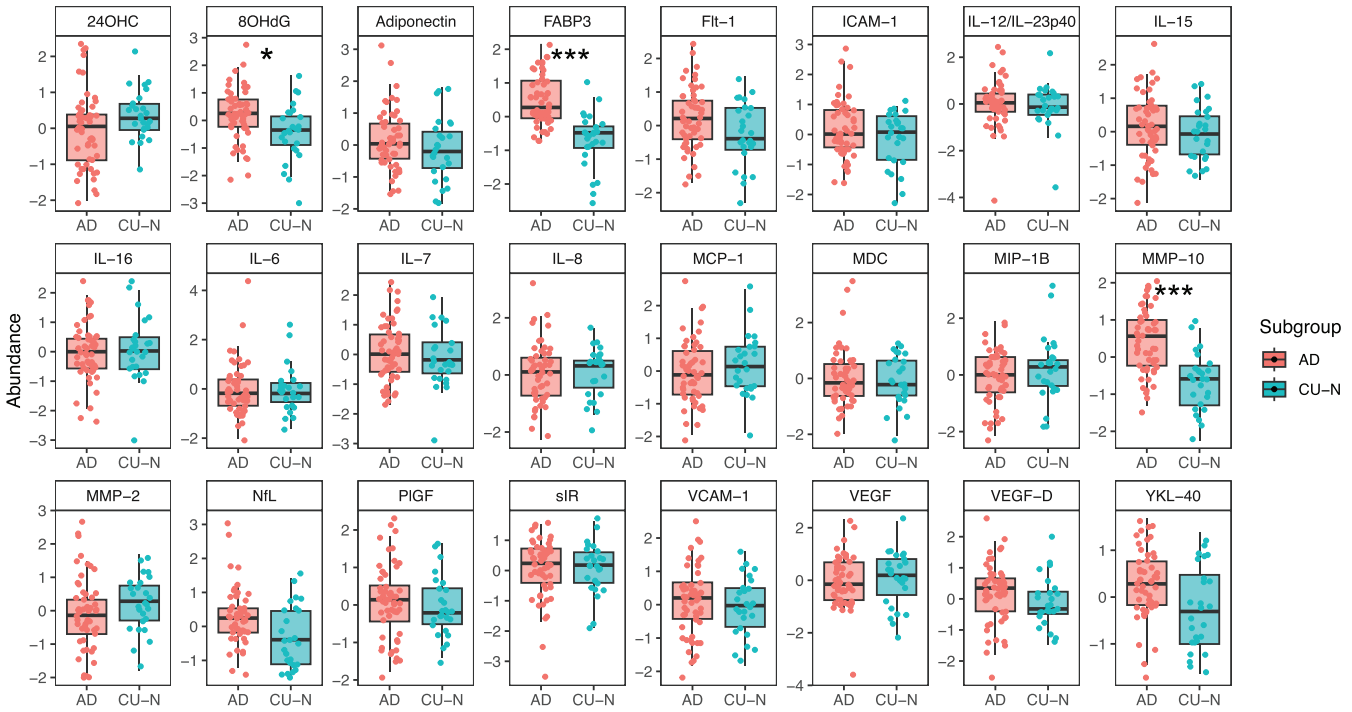


FIGURE 1 *Z*-score normalized levels of measured cerebrospinal fluid (CSF) biomarkers. Linear regression modeling adjusting for age and sex was used to determine differences between individuals with Alzheimer's disease (AD) and cognitively normal controls (CU-N). Presented *p*-values (*$p < 0.05$, ***$p < 0.0001$) were adjusted for multiple hypothesis testing using Benjamini-Hochberg correction.

number of *APOE* e4 carriers in the CU-N subgroup ($n = 5$) precluded further analysis in this group.

3.2 | Levels of individual CSF biomarkers and their intercorrelations

In order to assess the spectra of underlying pathophysiological processes in AD, the 25 biomarkers were categorized into one of four domains based on their predominant biological activities according to published literature (Table 2): (1) neurodegeneration (NfL, FABP3, YKL-40); (2) inflammation (IL-6, IL-7, IL-8, IL-12/23p40, IL-15, IL-16, MCP-1, MDC, MIP-1B); (3) vascular injury (Flt-1, PlGF, VEGF, VEGF-D, ICAM-1, VCAM-1, MMP-2, and MMP-10); and (4) metabolism (8-OHdG, 24-OHC, adiponectin, leptin, and soluble insulin receptor [sIR]). Using multivariate linear regression models with age and sex as covariates, we identified three markers, MMP-10 ($p < 0.0001$), FABP3 ($p < 0.0001$), and 8OHdG ($p < 0.05$), which were significantly elevated in participants with AD compared to CU-N (Figure 1). As the CU-N subgroup was heterogeneous, consisting of both HC and individuals with OND, we performed a sensitivity analysis comparing AD to a more homogeneous group consisting of only HC, which confirmed that MMP-10 and FABP3 levels were significantly elevated in AD ($p < 0.0001$ for both markers), while 8OHdG only remained significant before adjusting for multiple hypothesis testing (unadjusted $p < 0.05$; adjusted $p = 0.1$). Two additional markers, YKL-40 ($p < 0.01$) and VEGF ($p < 0.05$), were differentially expressed in participants with AD compared to HC in the sensitivity analysis (Figure S4).

While there were no significant differences between AD and CU-N on a group level for the remaining markers, we were interested in understanding heterogeneity in biomarker profiles in individual participants. Fifty percent of AD participants had increased biomarker levels in two or more domains (when classifying individuals as positive for a biomarker domain if they had at least one biomarker in that domain that exceeded 2 SD of the mean of the CU-N group), and 28% of AD participants were positive in one domain (Figure 2). The majority were positive for the vascular injury domain (61%), 37% for the inflammation domain, and 26% for the metabolism domain. A total of 41% were positive for the neurodegeneration domain. The CU-N group had fewer participants with positive biomarker domains compared to the AD group (54% vs. 78%; $p < 0.05$); specifically, fewer participants were positive for the vascular injury domain (31% vs. 61%; $p < 0.02$) or the neurodegeneration domain (14% vs. 39%; $p < 0.05$).

As many participants had increased levels of several biomarkers, we next explored the relationships between the measured biomarkers by generating a heat map of Pearson correlation coefficients in conjunction with hierarchical clustering (Figure 3). FABP3 and MMP-10, the two main biomarkers identified as differentially expressed in AD versus CU-N in univariate analyses, were found to be significantly correlated to each other ($r2 = 0.61$; $p < 0.0001$). FABP3 and MMP-10 were also highly correlated with NfL, YKL-40, and ICAM-1 ($r^2 > 0.55$; $p < 0.0001$), and FABP3 had additional significant associations with Flt-1 and IL-15 ($r^2 > 0.55$; $p < 0.0001$). The correlations across domains

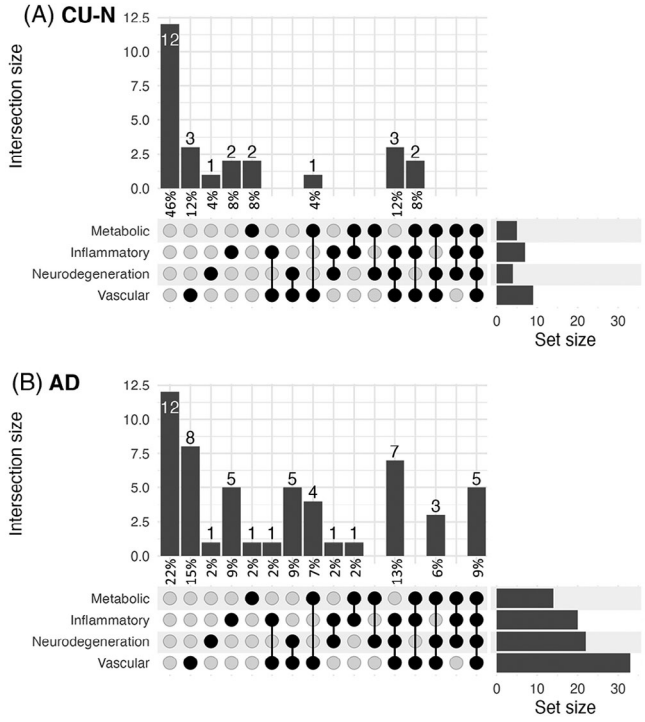


FIGURE 2 Upset plots showing the number (shown on top of individual bars) and frequency (shown below bars) of participants with increased levels of biomarkers in the different domains among cognitively normal controls (CU-N; top panel) and individuals with Alzheimer's disease (AD; bottom panel). A participant was considered positive for a particular domain if they had at least one biomarker exceeding 2 standard deviations of the mean of the CU-N group. Sample size: AD = 54; CU-N = 26.

raised the question of how well the initial categorization of biomarker domains based on the literature reflects the different pathophysiological processes of the biomarkers and their interactions in AD. Using unbiased hierarchical clustering, we noted that the biomarkers separated into four main clusters: (1) ICAM-1, VCAM-1, sIR, MMP-2, PlGF, VEGF, adiponectin, and 24-OHC; (2) NfL, MMP-10, FABP3, IL15, YKL-40, Flt-1, and VEGF-D; (3) IL-12/23p40, MDC, and IL-16; and (4) IL-8, MIP-1β, IL-6, MCP1, IL-7, and 8OHdG. These clusters appear largely to align with the different pathophysiological domains, with cluster (1) containing most metabolic biomarkers, cluster (2) reflecting neurodegenerative pathology, and clusters (3) and (4) containing predominantly inflammatory markers. Vascular biomarkers were present both in cluster (1) and (2) reflecting both that these clusters were more closely associated with each other than clusters (3) and (4), and the heterogeneity among the markers categorized as vascular markers, many of which have multiple biological activities.

To explore the degree to which the different biomarkers in the panel reflect AD pathology, we used linear regression analysis to correlate biomarker levels with established CSF AD biomarkers ($A\beta_{42/40}$ ratio, pTau-181, and tTau) within the AD sub-group (Figure S5). Notably, most biomarkers correlating with CSF AD biomarkers were present within the neurodegenerative cluster (cluster [2]). FABP3 and Flt-1 levels significantly predicted both pTau-181 ($r^2 = 0.16$ for both markers; $p < 0.005$) and tTau ($r^2 = 0.37$ and 0.26, respectively; $p < 0.0001$), while IL-15, MMP-10, NfL, YKL-40, and 8OHdG were also found to be weakly associated with tTau levels (r^2: 0.13–0.20; $p < 0.001$). No significant associations with an $r^2 > 0.1$ were found with the $A\beta_{42/40}$ ratio. This suggests that the mechanistic contributions of the metabolic and inflammatory markers in clusters (1), (3), and (4) may reflect alternative pathogenic processes than that of the classic AD biomarkers.

3.3 | Identifying a multi-analyte signature of AD

Given the degree of correlation between the individual biomarkers and their overlap between the diagnostic groups, we next sought to identify a subset of biomarkers that combined contribute the most towards differences between AD and CU-N. LASSO modeling with 1000 rounds of five-fold cross-validation selected FABP3, MMP-10, 24OHC, and MMP-2 as primary contributing markers to the signature, appearing in nearly 100% of trials (Figure 4A, B). We compared the performance of different model permutations of the top markers identified by the LASSO model using ROC to assess the ability to discriminate between AD and CU-N. A multi-analyte signature containing FABP3, MMP-2, MMP-10, 8OHdG, and 24OHC, exhibited the greatest discrimination between the groups, with a chi-square statistic of 13.59 ($p < 0.01$). Including age as a covariate did not significantly alter model outcomes.

We further characterized the signature biomarkers using PCA component analysis to examine the quality of the selected variables. The first component accounted for 42.8% of the overall variance in the dataset, driven by MMP-10, FABP-3, and MMP-2 (Figure 4C, D). The major contributors to component 2 were MMP2 and 24OHC, while 8OHdG was the main contributor to component 3. This analysis revealed two distinct sub-groups of positively correlated biomarkers with respect to component 2, with MMP-10, FABP3, and 8OHdG forming one group in opposition to a second group containing MMP-2, and 24OHC (Figure 4E).

4 | DISCUSSION

While defined by its signature Aβ and tau proteinopathies, AD is a complicated, heterogeneous disease and clinical syndrome driven by contributions of diverse risk factors and various underlying pathophysiological mechanisms. Amyloid and tau pathologies account for just a small proportion of the variance of global cognition in old age[21] and poorly explain features such as age of onset, rates of progression, or diverse clinical symptomatology. We developed a panel of robust assays with high technical reliability to measure 25 CSF biomarkers across different domains of pathophysiological importance in AD.[16] Here, we used these to explore the degree to which neurodegeneration, vascular injury, inflammation, and metabolic derangements are dysregulated in AD with the goal to identify a multi-analyte panel to more comprehensively profile the different pathophysiological domains and better understand the heterogeneous contributions in AD.

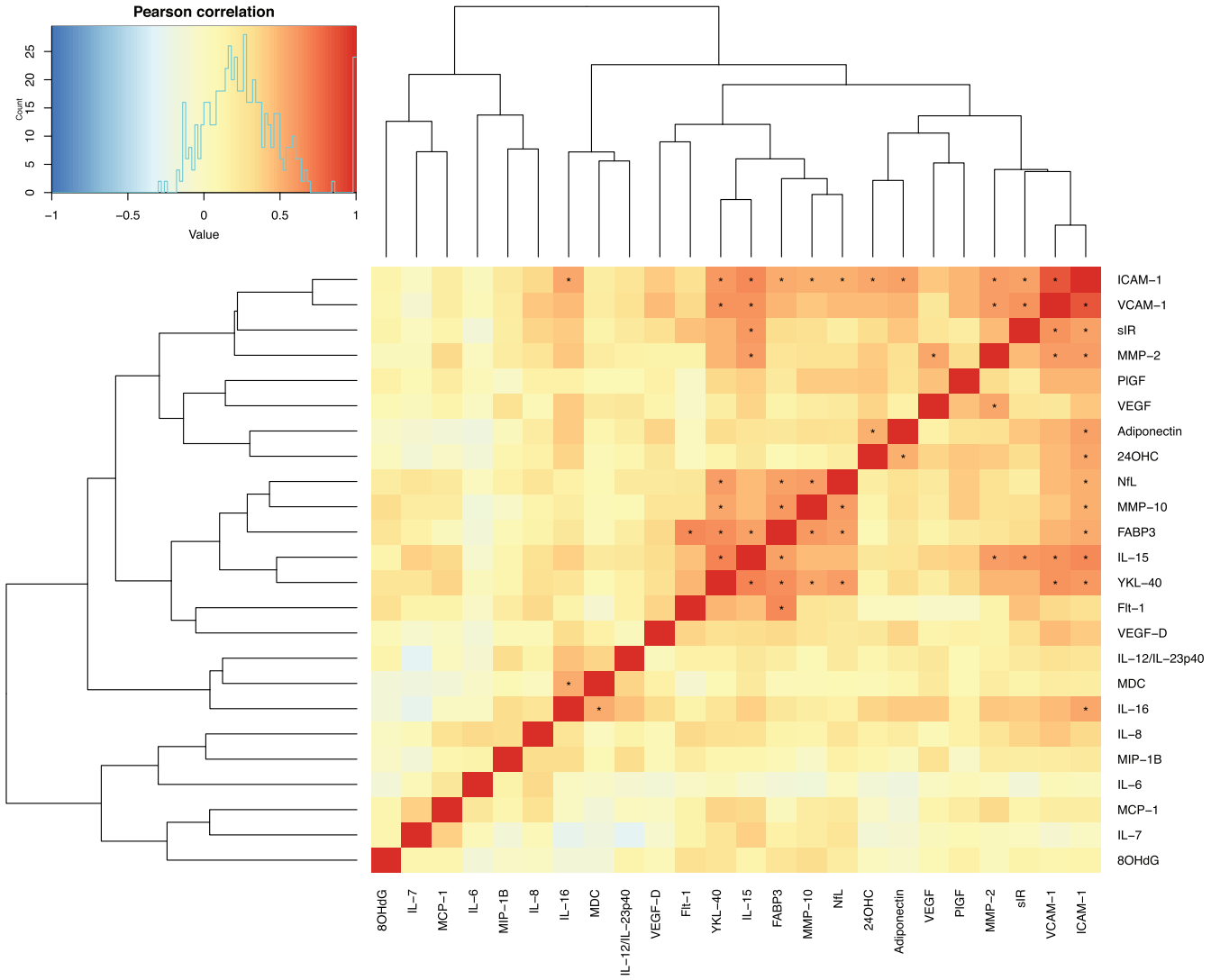
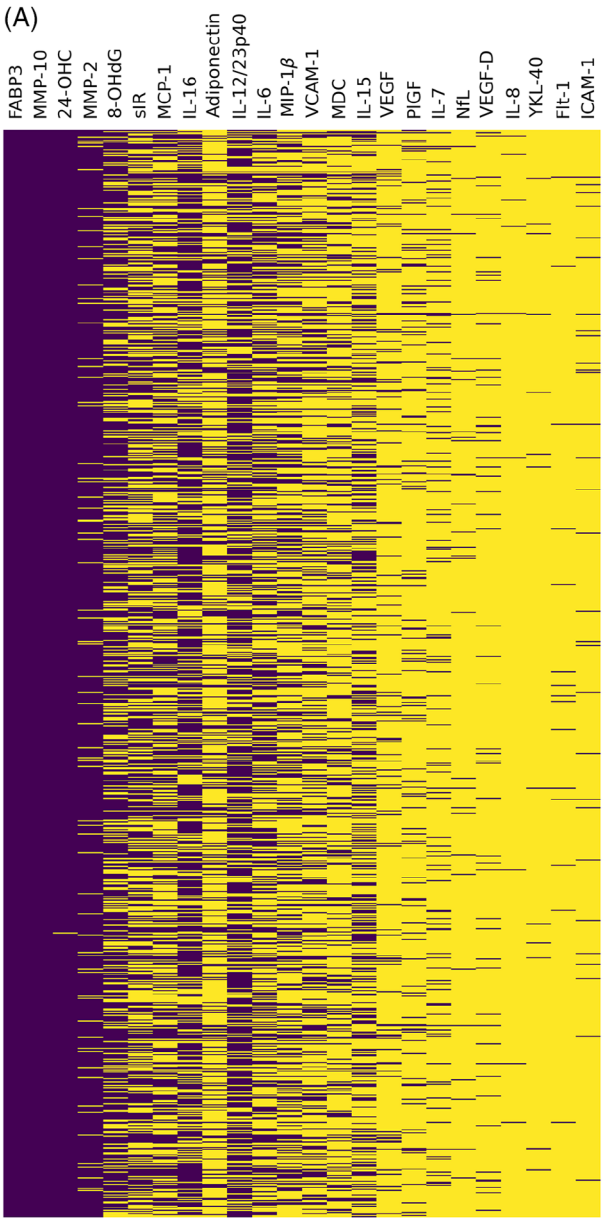


FIGURE 3 Heat map of Pearson correlation values with hierarchical clustering across analytes for all study participants ($N = 80$) coupled with dendrogram tree showing relationships between analytes. Significant correlations ($p < 0.05$) marked with an asterisk.

We noted that three biomarkers, MMP-10, FABP3, and 8OHdG, were increased in individuals with AD compared to CU-N in univariate analysis, three markers that previously have been implicated in AD.[22–27] MMP-10 is a zinc-dependent endopeptidase which can degrade extracellular matrices and other proteins.[28] It plays an important role in vascular remodeling during both development and in various vascular pathologic processes by degrading extracellular matrix components and thereby removing barriers to cell migration facilitating cell growth, proliferation, migration, and differentiation.[29] Increased CSF levels of MMP-10 have been associated with higher risk for progression from MCI to dementia and faster cognitive decline in AD.[22–25]

CSF levels of FABP3 are increased in AD, both in individuals with MCI/dementia and at the preclinical stage, and can predict progression of MCI to AD dementia.[30–34] FABP3 is detected at high levels in cortical neurons[35] and CSF levels are increased after trauma to the brain such as acute stroke, traumatic brain injury, and Creutzfeldt-Jakob disease likely through release by damaged cells, making FABP3 a putative biomarker for neuronal damage.[36] However, CSF levels of FABP3 may not simply reflect generalized neuronal degeneration in AD as levels are associated with Aβ pathology and risk factors associated with AD, predict development of AD in healthy individuals, and correlate with CSF tau and apolipoprotein levels.[30,37–39] FABP3 is a fatty acid-binding protein facilitating intracellular transport of omega-6 polyunsaturated fatty acids (PUFAs), which participate in the regulation of the lipid composition of membranes, neurite formation, and synapse plasticity.[40] Increased CSF levels of FABP3 in AD may reflect lipid dyshomeostasis, an established feature in AD.[40] Changes in brain lipid composition can under pathologic conditions result in a range of pathophysiological changes present in AD such as blood-brain barrier dysfunction, abnormal processing of amyloid-beta precursor protein (APP), defects in protein clearance, unbalanced energy metabolism, and inflammation.[41]

8OHdG, or 8-hydroxy-2'-deoxyguanosine, is a widely used biomarker of oxidative stress which is generated by reactive oxygen species (ROS) that oxidize the guanine of DNA.[42] Oxidative


(A)
FABP3
MMP-10
24-OHC
MMP-2
8-OHdG
sIR
MCP-1
IL-16
Adiponectin
IL-12/23p40
IL-6
MIP-1β
VCAM-1
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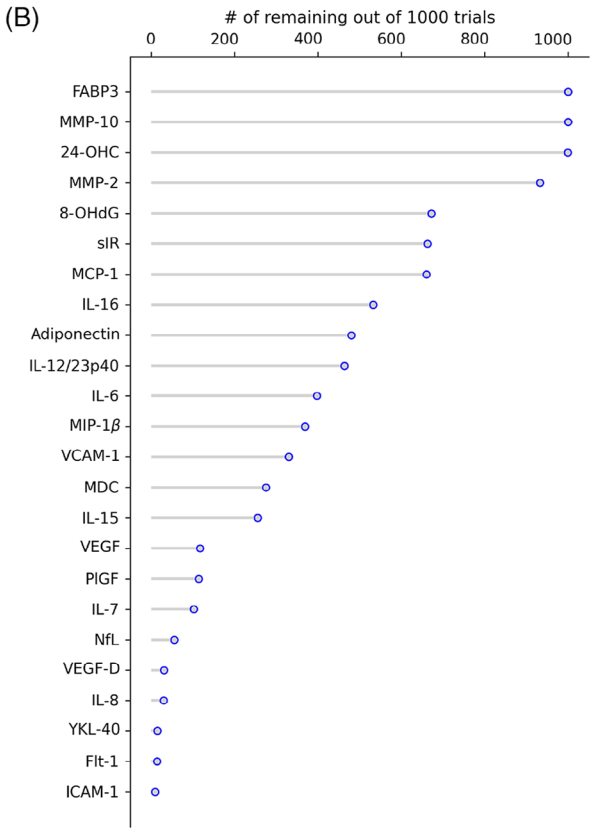
VEGF
PlGF
IL-7
NfL
VEGF-D
IL-8
YKL-40
Flt-1
ICAM-1


(B)
# of remaining out of 1000 trials
0
200
400
600
800
1000
FABP3
MMP-10
24-OHC
MMP-2
8-OHdG
sIR
MCP-1
IL-16
Adiponectin
IL-12/23p40
IL-6
MIP-1β
VCAM-1
MDC
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VEGF
PlGF
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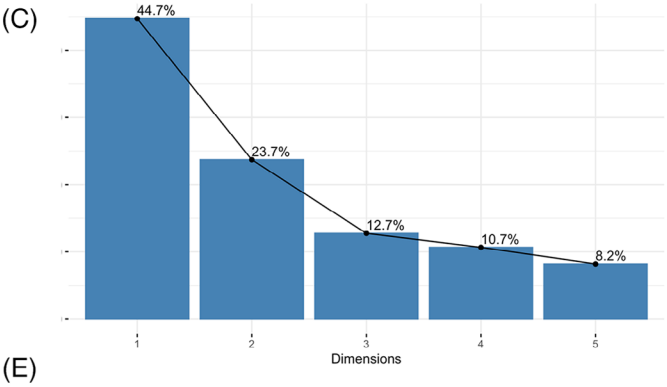
IL-8
YKL-40
Flt-1
ICAM-1


(C)
44.7%
23.7%
12.7%
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2
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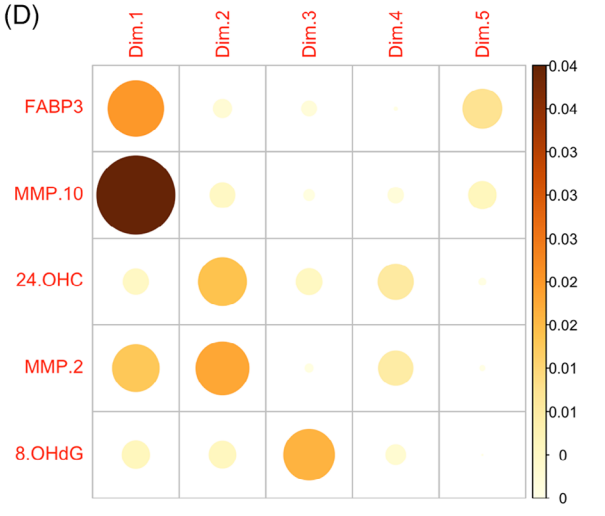
Dimensions


(D)
Dim.1
Dim.2
Dim.3
Dim.4
Dim.5
FABP3
MMP.10
24.OHC
MMP.2
8.OHdG
0.04
0.04
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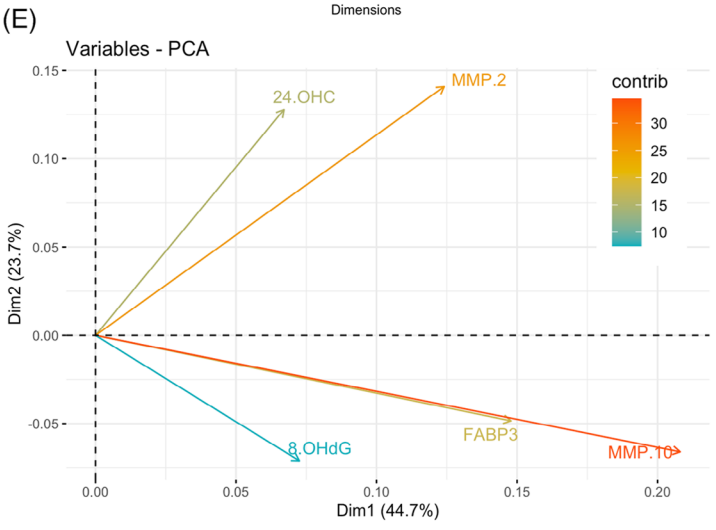
(E)
Variables - PCA
contrib
30
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24.OHC
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FABP3
MMP.10
8.OHdG
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Dim2 (23.7%)
Dim1 (44.7%)

stress is characteristic for AD and increased levels of 8OHdG and the related oxidation product 8-hydroxyguanosine (8OHG) are present in mitochondrial DNA (mtDNA) from postmortem brain tissue in AD[43] as well as in CSF from individuals with AD.[26,27] Oxidative stress is involved in Aβ- and tau-mediated neurotoxicity and contributes to synaptic loss, while also participating in abnormal Aβ accumulation and neurofibrillary tangle deposition, forming a vicious cycle promoting the initiation and progression of AD pathology.[44]

Large neuropathology studies have demonstrated that AD pathology commonly coexists with vascular pathology in individuals both with and without dementia.[15] More than half of the participants with AD had increased CSF levels of MMP-10, considered a vascular marker, in our study. In addition, 86% of the participants had increased CSF levels of one or more of the other vascular biomarkers under study (MMP-2, ICAM-1, VCAM-1, Flt1, PlGF, VEGF, and VEGF-D). The contribution of vascular pathology in the pathophysiology of AD and its clinical expression is still not clear, but there may be a synergistic effect between vascular and AD pathologies both when it comes to the development of tissue injury and cognitive impairment.[15] Using a simplified approach and ignoring contributions of other processes on cognitive decline in AD may lead to disappointing results in clinical outcomes in clinical trials. Even the recently successful anti-amyloid immunotherapies which target Aβ and effectively reduce its biomarker levels to normal in some people only seem to slow progression of dementia, not stop or reverse it. As with other complex disorders like cancer or heart failure, successful treatment of AD will almost certainly require multi-targeted therapy based on biomarker profiles.

A surprising finding when applying factor analysis to define a multi-analyte profile of AD was the contribution of 24OHC, a marker associated with metabolism. While included in the multi-analyte profile, 24OHC was not associated with AD in the univariate analysis. Analytes that do not differ significantly between two groups should not automatically be discounted, as statistical methods evaluating group averages largely disregard the information provided by the inter-individual variability of the analyte and assume that a disease will affect all individuals in a similar way. Processes affecting small subgroups resulting in marked differences between individuals in a group may not be sufficient to affect group means. More nuanced differences requiring much larger sample sizes to obtain power in univariate analysis may still contribute to the factor analysis.

Strengths of our study include the large panel of analytes highlighting the contribution of many different pathophysiological domains in AD and their relationships, that all assays were thoroughly validated resulting in excellent technical performance, and that a high-contrast cohort with molecular verification of the AD diagnoses was used. Limitations include the relatively small sample size which may limit the generalizability of the LASSO approach, that detailed clinical information about comorbidities and vascular risk factors were unavailable, and that samples were obtained when participants had established disease when downstream effects of neurodegeneration may contribute to the pathological abnormalities in addition to any causal mechanisms. Nevertheless, the median MMSE score in the AD group was 23.5 consistent with that most participants had relatively low cognitive impairment at the time of the sample collection. This is a stage of the disease when treatments are considered and individualized therapeutic approaches aimed at prominent pathological pathways would be needed.

In summary, we used a highly validated panel of 25 CSF biomarkers to better evaluate the heterogeneity of underlying pathophysiological processes in AD. We noted that, while AD was associated with increased levels of three biomarkers (MMP-10, FABP3, and 8OHdG) on a group level, half of all AD participants had increased levels of biomarkers belonging to at least two pathophysiological domains reflecting the diversity in AD. Interestingly, factor analysis showed that two markers of metabolism and oxidative stress (24OHC and 8OHdG) contributed independent information separate from MMP-10 and FABP3, associated with neurodegeneration and vascular injury. Better understanding of the heterogeneity among individuals with AD will be crucial for optimizing personalized treatment strategies aimed at specific mechanisms contributing to the disease process on an individual level and at different phases of the disease.

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CONFLICT OF INTEREST STATEMENT

Pia Kivisäkk and Steven E. Arnold are named as co-inventors of a US patent application related to neurological biomarker assays that is jointly held by Massachusetts General Hospital and Meso Scale Diagnostics. BC has received grant funding from Ono Pharmaceutical. SEA has received advisory board or consulting fees from Allyx Therapeutics, Bob's Last Marathon, Boyle Shaughnessy Law, Daewoong Pharmaceutical Co., Eisai, Jocasta Neuroscience, Quince Therapeutics (formerly

FIGURE 4 (A-B) Least absolute shrinkage and selection operator (LASSO) modeling with 1000 rounds of five-fold cross-validation was performed to identify a subset of biomarkers separating Alzheimer's disease (AD) and cognitively normal controls (CU-N). (C) Principal components of principal component analysis (PCA) analysis. The first four components account for 86.2% of variance. (D) FABP3, MMP-10, 24OHC, MMP-2, and 8OHdG were identified as the key contributors in the final diagnostic model. (E) The projection of FABP3, MMP-10, 24OHC, MMP-2, and 8OHdG to the top two principal components. The length of projection implies the representation of the variable on the principal components. Positive-correlated variables point to the same side of the plot, while negative-correlated variables point to opposite sides of the graph.

Cortexyme), Risen Pharmaceutical Technology, and Sage Therapeutics. B.A.T., C.Y.W., E.K., M.B.G., and H.H.D. have no declarations of interest related to to the contents of the work presented herein. Author disclosures are available in the supporting information.

CONSENT STATEMENT

This study was approved by the Institutional Review Boards of Mass General Brigham and University of Pennsylvania and all study participants or their assigned surrogate decision makers provided written informed consent for use of their samples in biomarker research.

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SUPPORTING INFORMATION

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

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