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FEATURED ARTICLE

Large-scale plasma proteomic profiling identifies a high-performance biomarker panel for Alzheimer's disease screening and staging

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Abstract

Introduction: Blood proteins are emerging as candidate biomarkers for Alzheimer's disease (AD). We systematically profiled the plasma proteome to identify novel AD blood biomarkers and develop a high-performance, blood-based test for AD.

Methods: We quantified 1160 plasma proteins in a Hong Kong Chinese cohort by high-throughput proximity extension assay and validated the results in an independent cohort. In subgroup analyses, plasma biomarkers for amyloid, tau, phosphorylated tau, and neurodegeneration were used as endophenotypes of AD.

Results: We identified 429 proteins that were dysregulated in AD plasma. We selected 19 "hub proteins" representative of the AD plasma protein profile, which formed the basis of a scoring system that accurately classified clinical AD (area under the curve = 0.9690–0.9816) and associated endophenotypes. Moreover, specific hub proteins exhibit disease stage-dependent dysregulation, which can delineate AD stages.

Discussion: This study comprehensively profiled the AD plasma proteome and serves as a foundation for a high-performance, blood-based test for clinical AD screening and staging.

KEYWORDS
Alzheimer's disease, biomarker panel, diagnosis, disease staging, neurodegenerative disease, plasma proteome, prognosis

1 | INTRODUCTION

Evaluating the ATN biomarkers of Alzheimer's disease (AD) in the brain, including amyloid beta (Aβ) deposition ("A"), neurofibrillary tangles (pathologic tau, "T") and neurodegeneration ("N") requires invasive cerebrospinal fluid sampling for protein measurement and/or costly imaging by positron emission tomography (PET), greatly restricting their utility for population-scale AD screening.[1–3] The recent discovery of blood-based AD biomarkers (i.e., plasma A$\beta_{42/40}$ ratio, tau/phosphorylated tau [p-tau], and neurofilament light polypeptide [NfL]) raises the possibility of an alternative, less-invasive, blood-based test for AD.[4–7] In particular, plasma p-tau181 and p-tau217 accurately classify AD and are associated with AD-specific brain pathologies including tau phosphorylation and Aβ deposition.[6,7] Moreover, cross-sectional and longitudinal studies have demonstrated that plasma p-tau and NfL can indicate disease progression.[5,7–9] Nonetheless, given their relatively constant changes during AD progression,[5,7–11] these blood biomarkers might not have clear stage-specific patterns to define AD stages. Moreover, as a few pilot screening studies identified alternative AD-associated blood proteins with predictive value,[12–17] it remains unclear whether the existing AD blood biomarkers sufficiently capture the complete signatures of the AD blood proteome. Therefore, comprehensive protein profiling is needed to clarify the protein signatures of AD blood and delineate the disease pathways and stages.

Recent advances in ultrasensitive and high-throughput protein measurement technologies have enabled large-scale proteomic profiling of the blood,[18,19] which have been widely adopted to study cardiovascular diseases and aging, consequently identifying novel biomarkers and providing biological annotations for disease stages.[20,21] Accordingly, in this study, we used proximity extension assay (PEA) technology to systematically evaluate the protein profiles of AD plasma. Specifically, in a Hong Kong Chinese AD cohort ("discovery cohort" hereafter), consisting of 106 patients with AD and 74 healthy controls (HCs) for whom demographic data, cognitive measures, brain region volumes, and plasma biomarker levels (i.e., A$\beta_{42/40}$ ratio, tau, p-tau181, and NfL) were available (Table S1 in supporting information), we quantified 1160 plasma proteins and revealed 429 plasma proteins that were dysregulated in patients with AD. We further identified a 19-protein biomarker panel representative of the plasma proteomic signature of AD and validated its high accuracy for classifying AD and associated endophenotypes in an independent cohort. In addition, we showed that certain plasma biomarker proteins are dysregulated in specific stages of AD. Thus, we determined a comprehensive profile of the AD plasma proteome and established a high-performance plasma biomarker panel for AD, which constitutes a critical foundation for developing a blood-based test for AD screening and staging.

2 | METHODS

2.1 | Subject recruitment

The discovery cohort comprised 180 Hong Kong Chinese people ≥60 years old, including 106 patients with AD and 74 HCs who visited the Specialist Outpatient Department of the Prince of Wales Hospital of the Chinese University of Hong Kong from April 2013 to February 2018. The clinical diagnosis of AD was established on the basis of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5).[22] All participants underwent medical history assessment, clinical assessment, cognitive and functional assessment using the Montreal Cognitive Assessment (MoCA), and neuroimaging assessment by magnetic resonance imaging (MRI).[23,24] Participants with any significant neurological disease other than AD or a psychiatric disorder were excluded. Age, sex, years of education, medical history, history of cardiovascular disease (i.e., heart disease, hypertension, diabetes mellitus, and hyperlipidemia), and white blood cell count were recorded. This study was approved by the Prince of Wales Hospital of the Chinese University of Hong Kong as well as the Hong Kong University of Science and Technology. All participants provided written informed consent for both study participation and sample collection.

The validation cohort comprised 97 Hong Kong Chinese people ≥60 years old, including 36 patients with AD and 14 HCs who

visited Queen Elizabeth Hospital from February 2018 to March 2020 as well as 47 HCs who visited the Community CareAge Foundation or Haven of Hope Christian Service from October 2019 to January 2020. The participants recruited from Queen Elizabeth Hospital underwent medical history assessment, clinical assessment, cognitive and functional assessment using the MoCA, and neuroimaging assessment by MRI.[23,24] The clinical diagnosis of AD was based on the US National Institute on Aging and Alzheimer's Association (NIA-AA) workgroup 2011 revised criteria.[1,25] Participants with any significant neurological disease other than AD or a psychiatric disorder were excluded. The participants recruited from the Community CareAge Foundation or Haven of Hope Christian Service, representing population-level HCs, underwent medical history assessment as well as cognitive and functional assessment using the MoCA.[23,24] Age, sex, years of education, and medical history were recorded. This study was approved by Queen Elizabeth Hospital, the Community CareAge Foundation, Haven of Hope Christian Service, and the Hong Kong University of Science and Technology. All participants provided written informed consent for both study participation and sample collection.

The demographic data of both cohorts and the details of sample collection are presented in Table S1 and the Supplementary Methods section in supporting information.

2.2 | Plasma protein measurement

The $A\beta_{42/40}$ ratio, tau, p-tau181, and NfL levels were measured in 350 μL plasma by Quanterix Accelerator Lab using the Quanterix NF-light SIMOA Assay Advantage Kit (103186), the Neurology 3-Plex A Kit (101995), or the P-Tau 181 Advantage V2 Kit (103714) where appropriate. The levels of 1160 proteins were quantified in 20 μL plasma by Olink Proteomics using PEA technology (Supplementary Methods). The levels of the assayed plasma proteins are presented in normalized protein expression units. Selected plasma proteins were further validated by enzyme-linked immunosorbent assay (ELISA; Supplementary Methods).

2.3 | Plasma proteome–AD association analysis

Prior to analysis, the proteomic data were subjected to rank-based normalization using the rntransform() function from the R GenABEL package (v1.8). AD-associated alterations in the plasma proteome were determined according to the associations between the normalized protein levels and AD phenotypes after adjusting for age, sex, history of cardiovascular disease (CVD; i.e., heart disease, hypertension, diabetes mellitus, and hyperlipidemia), and population structure (i.e., the top five principal components [PCs] obtained from the results of principal component analysis of whole-genome sequencing data; Supplementary Methods) using the following linear regression model:

Normalized protein level

$$\sim \beta_1 AD + \beta_2 Age + \beta_3 Sex + \beta_i CVD_i + \beta_j PC_j + \varepsilon$$

RESEARCH IN CONTEXT

1. **Systematic review**: A literature review was conducted using traditional sources (e.g., PubMed). Although the blood-based ATN biomarkers are good candidates for classifying Alzheimer's disease (AD), given the complexity of the blood system, it remains unclear whether these biomarkers sufficiently capture the whole signature of AD blood proteome. Moreover, given the lack of stage-specific AD biomarkers, the biological definitions of AD stages remain unclear. Therefore, comprehensive profiling of the AD plasma proteome might help clarify the protein signatures of AD blood and provide insights into disease staging.

2. **Interpretation**: This study systematically investigated the AD plasma proteome. It verified many known AD-associated plasma proteins across ethnic groups and revealed new candidate biomarkers of AD. It demonstrated that a composite biomarker panel can achieve highly accurate AD classification and delineate stages.

3. **Future directions**: The clinical application of the protein panel will be strengthened by prospective longitudinal studies of mild cognitive impairment and AD. Moreover, studies of other neurodegenerative diseases will provide additional support for the specificity of the panel.

where β is the weighted coefficient for the corresponding factors and ε is the intercept of the linear equation. The plasma proteins with a false discovery rate-adjusted P-value less than 0.05 were considered AD-associated plasma proteins.

2.4 | Correlation network analysis

Pairwise correlations between plasma proteins were determined by calculating Pearson's correlation coefficients (r) using the cor() function in R. The AD-associated plasma proteins in the correlation matrix were clustered by hierarchical agglomerative clustering using the dist() function, with the Euclidean distance matrix and the hclust() function according to the complete linkage method ($h = 4$) in R, which yielded 19 major protein clusters. The top AD-associated plasma protein (i.e., the one with the lowest P-value) in each cluster was designated the "hub" protein.

2.5 | Gene Ontology and cell-type enrichment analyses

Gene Ontology (GO) analysis of candidate plasma proteins was performed using DAVID Bioinformatics Resources.[26,27] An enrichment analysis of the cell types in the peripheral blood system was conducted with reference to the transcriptome profiles of the corresponding cell

types from the BLUEPRINT Genomatix Browser[28] (Supplementary Methods).

2.6 | Plasma proteome-based AD classification

Only individuals in whom the 19-protein biomarker panel and plasma ATN biomarkers were detectable (i.e., above the lower limit of detection; $n = 172$ and 97 for the discovery and validation cohort, respectively) were included in subsequent analyses. The accuracy of AD classification based on individual plasma proteins was evaluated by calculating areas under the curve (AUCs) using the auc() function from the R pROC package. The cutoffs for individual proteins as well as the corresponding sensitivity and specificity were determined using the optimal.cutpoints() function and the Youden method from the R OptimalCutpoints package (Supplementary Methods).[29] For AD classification using multiple candidate factors, three models were established on the basis of age, sex, and the plasma levels of (1) the $A\beta_{42/40}$ ratio, tau, and NfL; (2) the 19-protein biomarker panel; and (3) the $A\beta_{42/40}$ ratio, tau, and NfL plus the 19-protein biomarker panel. The classification accuracy between two models was compared by calculating AUCs using the auc() function from the R pROC package using the DeLong method[30] (Supplementary Methods).

2.7 | Calculation of AD classification scores

Individual AD classification scores were calculated using a linear regression model that included age, sex, and the plasma levels of the 19-protein biomarker panel as candidate factors:

$$\text{Individual AD classification score}$$
$$= \frac{1}{1 + e^{-(\beta_1 \text{Age} + \beta_2 \text{Sex} + \beta_i \text{Hub protein}_i + \varepsilon)}}$$

where the weighted coefficient (β) of the candidate factors and the intercept (ε) were determined by fitting the corresponding factors and phenotype information into a logistic regression model[31] (Supplementary Methods). The AD severity levels were designated according to the distribution of AD classification scores: individuals with scores <0.3, 0.3–0.8, and > 0.8 were classified as normal, having mild AD, and having severe AD, respectively.

2.8 | Statistical analysis and data visualization

The significance of the associations between AD-associated endophenotypes and candidate plasma proteins as well as the AD severity levels determined by the 19-protein model was determined by linear regression analysis. The level of significance was set to $P < 0.05$. For data visualization, the heatmap.2() function from the R gplots package (v3.0.1.1) was used to generate a heatmap of the top 15 dysregulated plasma proteins and the correlation matrix of candidate plasma proteins. In addition, a volcano plot was generated using the plot() function in R. Furthermore, an edge-weighted network plot was generated using the R visNetwork package (v2.0.9), and the plot.roc() function from the R pROC package (v1.15.3) was used to generate the ROC curves for the classification models. All other statistical plots were generated using GraphPad Prism v8.0 (GraphPad Software).

3 | RESULTS

3.1 | Identification of differentially expressed plasma proteins in patients with AD

The plasma $A\beta_{42/40}$ ratio, tau level, and NfL level—collectively termed the plasma ATN biomarkers—are the best-known proteins that are altered in the blood of patients with AD.[4–6] Therefore, we measured their levels in the discovery cohort using the SIMOA digital biomarker detection platform before screening for AD-associated proteins. Consistent with previous findings in populations of European descent,[32–34] patients with AD in the discovery cohort had a lower plasma $A\beta_{42/40}$ ratio and a higher plasma NfL level compared to the HCs ($P < 0.001$, Figure S1A, C in supporting information), whereas plasma tau levels did not differ significantly between groups ($P = 0.830$, Figure S1B). These results show that the plasma ATN biomarkers exhibit consistent changes in Chinese patients with AD.

We subsequently performed PEA to identify which plasma proteins are differentially expressed in AD. Among the 1160 assayed plasma proteins, 429 were differentially expressed in patients with AD, including 61 significantly upregulated proteins and 368 significantly downregulated proteins (false discovery rate [FDR]-adjusted $P < 0.05$; Figure 1A, B and Table A1 in supporting information). We cross-validated the accuracy of the PEA-based quantification of CASP-3, CD8A, and NfL using the ELISA and SIMOA platforms (Figure S2 in supporting information). Moreover, meta-analysis showed that 77 of the 429 AD-associated plasma proteins have been reported in populations of European descent, 56 of which were consistently altered in patients with AD (Table A1 in supporting information).[12-17, 35-42] Specifically, to examine whether consistent changes can be observed in the prodromal stage of AD-related dementia in populations of European descent, we compared our results to a pilot proteomics study in a Swedish mild cognitive impairment (MCI) cohort (i.e., BioFINDER), which assayed 270 plasma proteins using the same PEA-based platform in $A\beta+$ cognitively normal participants as well as $A\beta-$ and $A\beta+$ participants with MCI (Figure S3A in supporting information).[17] The changes in the levels of most plasma proteins measured in both studies exhibited similar trends (maximum $r^2 = 0.31$, $P < 0.0001$; Figure S3B in supporting information). Further examination of the AD-associated plasma proteins identified herein revealed stronger correlations between the changes in the protein levels in the two cohorts (maximum $r^2 = 0.49$, $P < 0.0001$; Figure S3C). Taken together, these results support that the AD-associated plasma proteins identified herein are highly reproducible across protein-detection platforms, ethnic groups, and disease stages.
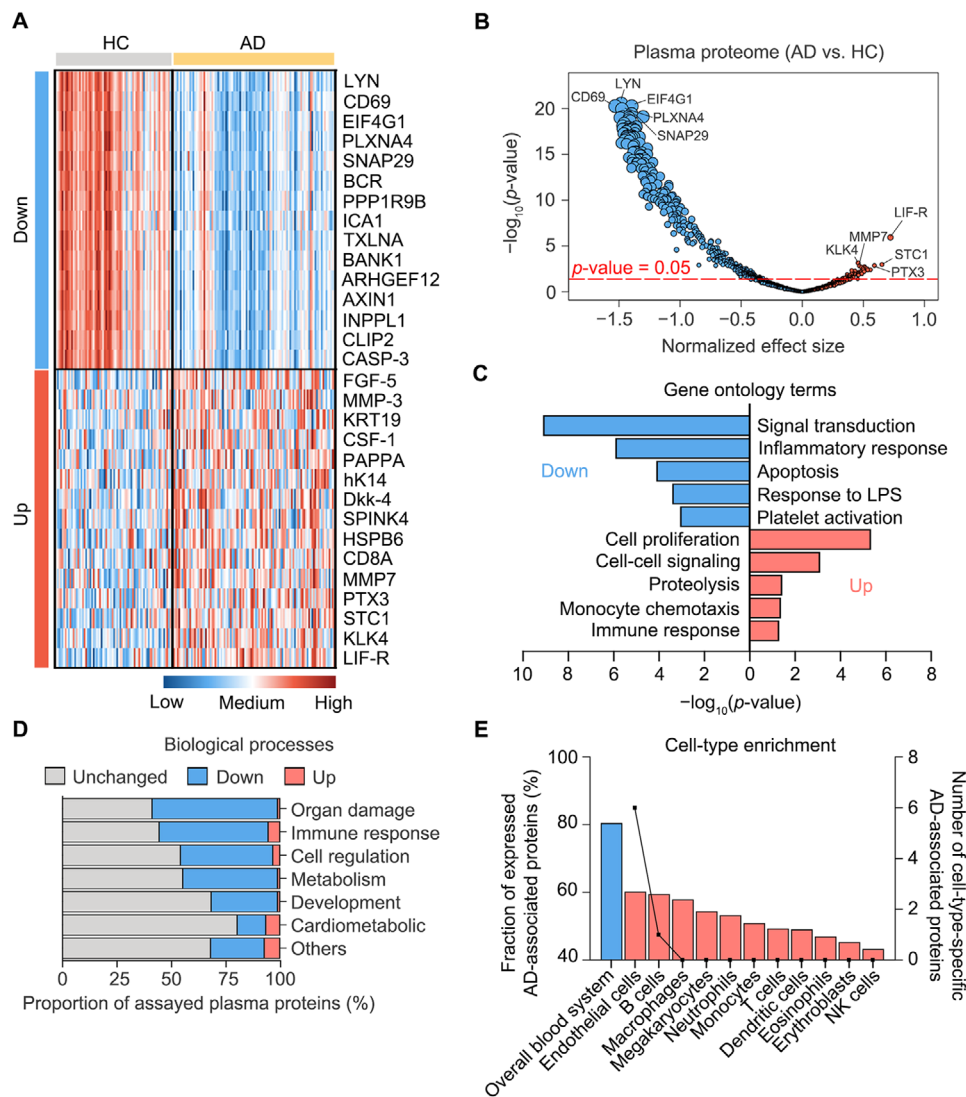
FIGURE 1 Alteration of the plasma proteome in patients with Alzheimer's disease (AD). A, Heatmap of the levels of the top 15 down- and upregulated AD-associated plasma proteins (i.e., those with the lowest P-values) in healthy controls (HCs) and patients with AD. B, Volcano plot showing the associations among 1160 plasma proteins. Blue and red dots indicate proteins in patients with AD that were down- or upregulated compared to HCs, respectively. Dot size is proportional to the P-value (in $\log_{10}$ scale), and the top five down- and upregulated plasma proteins are labeled. C, Representative Gene Ontology (GO) terms of the AD-associated plasma proteins. The GO terms of the down- and upregulated plasma proteins are indicated in blue and red, respectively. D, Proportions of the unchanged (gray), downregulated (blue), and upregulated (red) plasma proteins in each biological category. E, Cell sources of AD-associated plasma proteins. The left and right Y-axes denote the fractions of expressed AD-associated plasma proteins (bars) and numbers of cell-type-specific, AD-associated plasma proteins (black line), respectively, among the 11 major peripheral blood cell types (red) or in the overall peripheral blood system (blue). NK cells, natural killer cells; LPS, lipopolysaccharide

Next, we identified enriched functional categories among the 429 AD-associated plasma proteins by performing GO analysis. The upregulated proteins in patients with AD are mostly associated with cell proliferation (FDR-adjusted $P = 3.4E−6$) or chemotaxis (FDR-adjusted $P = 3.9E−2$), whereas the downregulated proteins are mostly involved in inflammatory response (FDR-adjusted $P = 1.2E−6$) or apoptosis (FDR-adjusted $P = 7.7E−5$; Figure 1C and Table A2 in supporting information). Subsequent classification of these AD-associated plasma proteins revealed that pathways related to organ damage and immune response are particularly dysregulated in AD ($P < 0.001$); more than 50% of assayed proteins from these pathways were significantly reduced in AD plasma (Figure 1D). Moreover, cell-type enrichment analysis revealed that ≈81% of the 429 AD-associated plasma proteins are expressed by peripheral blood cells; in particular, six of the AD-associated plasma proteins—MMP1, CDH5, LAMA4, BGN, SNCG, and LIF-R—are exclusively expressed by endothelial cells (Figure 1E, Table S2 and A3 in supporting information). Taken together, our findings identify a plasma proteomic signature of AD.

3.2 | A 19-protein biomarker panel represents the overall AD plasma proteome

Co-expression network analysis or GO analysis can classify many functionally related plasma proteins into clusters or pathways, enabling the selection of a subset of proteins that is representative of the overall plasma proteome.[43] To determine the minimum number of proteins required to sufficiently represent the plasma proteomic profile of patients with AD, we performed correlation network analysis to examine the coregulation patterns of the 429 AD-associated plasma proteins followed by hierarchical agglomerative clustering analysis on the resultant correlation matrix. This yielded 19 distinct clusters of AD-associated plasma proteins (Figure 2A), each associated with a distinct biological process and unique cell-type expression profile (Table S3 and Table A3 in supporting information). For example, cluster 3 comprises 12 upregulated proteins involved in cell adhesion-related pathways (FDR-adjusted $P = 1.0E-3$) that are mainly expressed by endothelial cells ($P = 1.1E-2$), whereas cluster 19 comprises 134 downregulated proteins involved in apoptosis (FDR-adjusted $P = 1.4E-3$) that are mainly expressed by megakaryocytes ($P = 3.0E-4$) and B cells ($P = 2.0E-3$; Table S3). Given that the proteins within each cluster are mostly coregulated (Figure 2B), we subsequently designated a "hub" protein for each of the 19 clusters by selecting the protein most strongly associated with AD (i.e., the one with the lowest P-value; Figure 2C and Table S4 in supporting information). Of note, correlation analysis of the 19 hub proteins with the plasma ATN biomarkers revealed that 10 hub proteins were correlated with the A$\beta_{42/40}$ ratio, tau level, or NfL level, whereas the remaining 9 proteins were not correlated with any of them (**Figure 2D** and Table A4 in supporting information). Thus, we identified 19 plasma hub proteins that capture both the ATN-dependent and ATN-independent changes in the AD plasma proteome and are representative of the overall plasma proteomic profile of patients with AD.

3.3 | An integrative model based on the 19-protein biomarker panel accurately distinguishes AD

The plasma ATN biomarkers are the best characterized and most widely studied blood protein candidates for AD classification.[32–34] Accordingly, in the discovery cohort, AD classification based on the plasma ATN biomarkers had a maximum accuracy of 87.35% (Figure 3A, B and Table S5 in supporting information). Given that the 19 hub proteins mentioned above are consistently altered in AD plasma irrespective of patient age, sex, AD drug usage, or apolipoprotein E (APOE) ε4 genotype (Figure S4A and Table A5 in supporting information), we examined whether they could also be used to classify AD. Therefore, we established an integrative model for AD classification based on the levels of the 19 hub proteins (see Sections 2.6 and 2.7), adjusting for age and sex[21] (Table S4). We then applied this 19-protein model to generate an AD classification score for each individual, which accurately distinguished patients with AD from HCs (AUC = 0.9816; Figure 3A–C). Moreover, the model classified AD with similar accu-
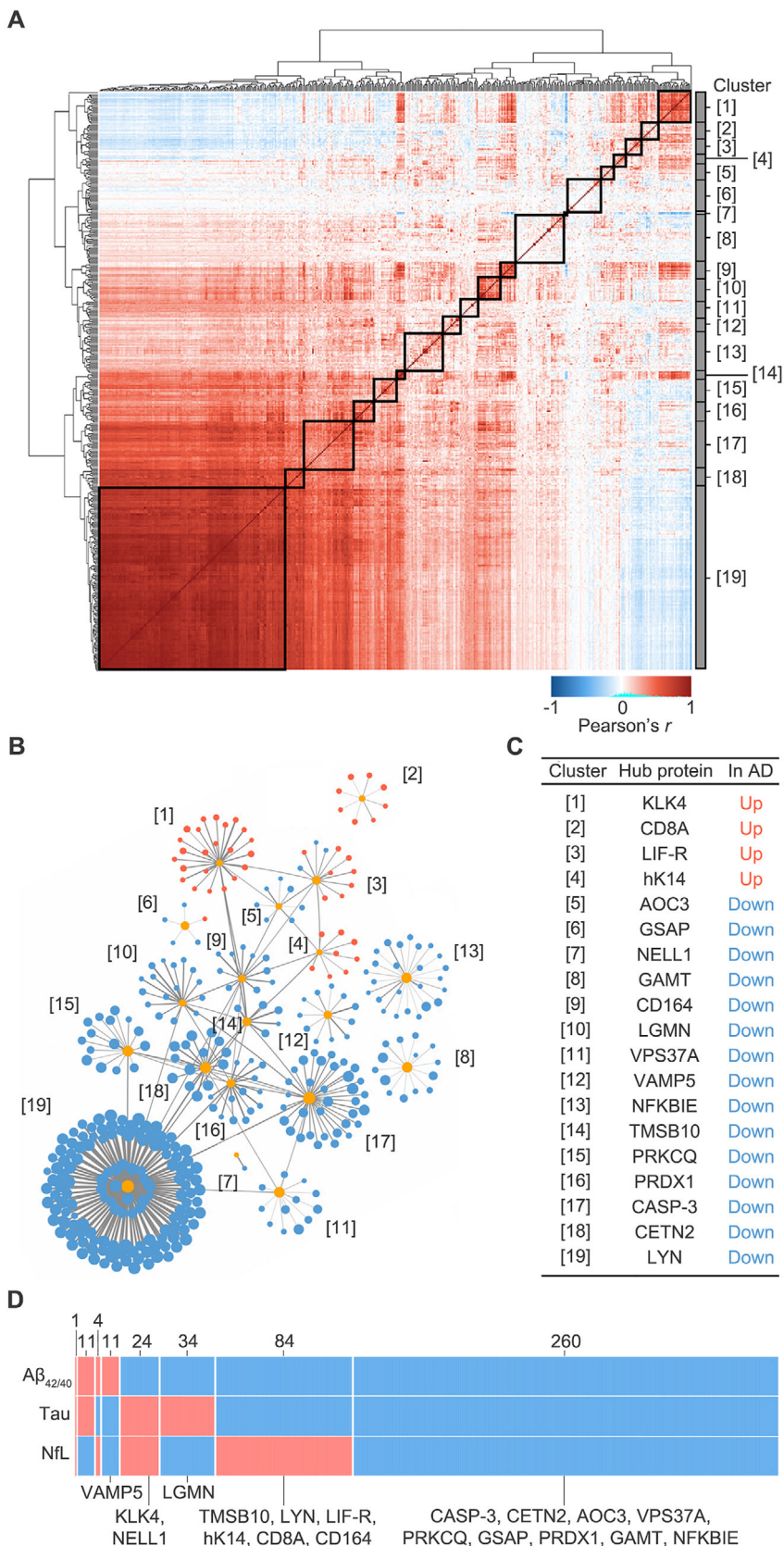
racy in the discovery cohort when stratified by sex, age, AD drug usage, or APOE ε4 genotype (Figure S4B–E). Of note, its performance was superior to that of the model integrating the plasma ATN biomarkers (19 proteins vs. ATN: $P < 0.001$; Figure 3A, B). Moreover, integrating these three plasma ATN biomarkers into the 19-protein model did not improve the accuracy of AD classification (AUC = 0.9891, 19 proteins vs. ATN + 19 proteins: $P = 0.456$; Figure 3A, B). This is consistent with our finding that the 19-protein panel captures both ATN-dependent and ATN-independent changes in AD plasma (Figure 2D), suggesting that our 19-protein model can accurately distinguish patients with AD from HCs.
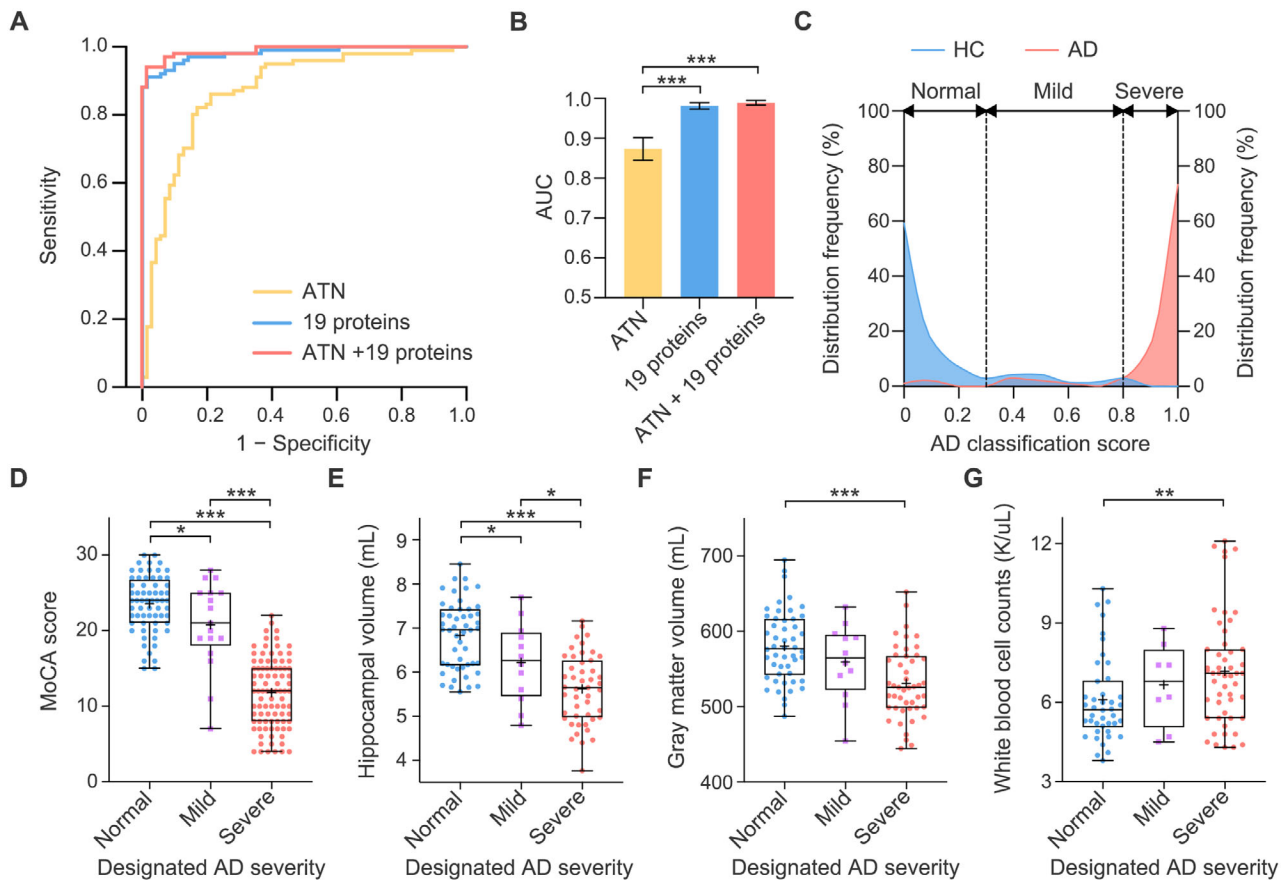
Interestingly, 7 of the 19 plasma hub proteins were significantly correlated with cognitive performance (Table S6 in supporting information), suggesting potential roles in AD progression. Concordantly, the classification accuracy of the 19-protein model was correlated with the MoCA scores of patients with AD (Figure S5 in supporting information). Therefore, we subsequently examined the capability of the 19-protein model to predict AD-associated endophenotypes. Most HCs had AD classification scores less than 0.3, whereas patients with AD had AD classification scores greater than 0.8 (Figure 3C). Therefore, we classified individuals with scores < 0.3, 0.3–0.8, or > 0.8 as normal, mild AD, and severe AD, respectively. The designated severity levels were strongly correlated with cognitive performance (normal vs. severe: $P < 0.001$; Figure 3D) as well as decreases in hippocampal volume (normal vs. severe: $P < 0.001$; Figure 3E) and gray matter volume (normal vs. severe: $P < 0.001$; Figure 3F). Moreover, individuals classified as having severe AD exhibited elevated white blood cell count (normal vs. severe: $P < 0.01$; Figure 3G), suggesting more pronounced inflammation.[44] Taken together, these results demonstrate that our integrative model based on the 19-protein biomarker panel not only accurately distinguishes patients with AD from HCs but can also reflect AD-associated endophenotypes. Thus, our model could serve as the basis for developing a strategy for individual, high-performance AD screening and monitoring.

3.4 | Validation of the 19-protein biomarker panel in an independent cohort

Next, to validate whether the 19-protein panel can be used for high-performance AD screening, we measured the plasma concentrations of the 19 hub proteins in an independent "validation cohort" ($n = 36$ patients with AD, $n = 61$ HCs; Figure 4A and Table S1). Six proteins—KLK4 (Figure 4B), LIF-R (Figure 4C), CASP-3 (Figure 4D), NELL1 (Figure 4E), CD164 (Figure 4F), and LYN (Figure 4G; all $P < 0.05$)—were cross-validated, exhibiting significant alterations in patients with AD consistent with those observed in the discovery cohort. In addition, 10 other hub proteins exhibited trends consistent with the changes in the discovery cohort (Figure S6 and Table S7 in supporting information). Applying the same integrative model based on the plasma levels of the 19 hub proteins to the validation cohort resulted in highly accurate AD classification (AUC = 0.9690), which was again more accurate than the model integrating the plasma ATN biomarkers (AUC = 0.8871,

FIGURE 2 Identification of Alzheimer's disease (AD)-associated plasma hub proteins. A, Heatmap showing the pairwise correlations among AD-associated plasma proteins. Each row and column represent 1 of the 429 AD-associated plasma proteins. Red and blue indicate positive and negative correlations between protein pairs, respectively. Black squares denote the 19 protein clusters based on hierarchical clustering, and numbers in brackets on the right indicate the cluster number. B, Correlation network plot of the AD-associated plasma hub proteins. Numbers in brackets adjacent to the clusters indicate the corresponding cluster number. Dot size is proportional to the P-value (in the $\log_{10}$ scale). Yellow dots denote the 19 plasma hub proteins, and blue and red dots indicate proteins in patients with AD that were down- and upregulated compared to healthy controls (HCs), respectively. Edges represent pairwise correlations between individual AD-associated plasma proteins and plasma hub proteins, and line thickness is proportional to the correlation coefficient. C, The 19 plasma hub proteins identified in each cluster. Red and blue indicate the up- and downregulated plasma hub proteins, respectively. D, Correlations between 429 AD-associated plasma proteins and the plasma ATN biomarkers (i.e., amyloid-beta [Aβ]$_{42/40}$ ratio, tau level, and neurofilament light polypeptide [NfL] level). Each row represents a plasma ATN biomarker, and each column represents 1 of the 429 AD-associated plasma proteins. Red and blue indicate significant ($P < 0.05$) and nonsignificant correlations ($P > 0.05$) between the protein pairs, respectively. Squares denote categories based on the correlations with the plasma ATN biomarkers, the corresponding numbers above indicate the number of plasma proteins in each category, and the plasma hub proteins are listed below

Cluster	Hub protein	In AD
[1]	KLK4	Up
[2]	CD8A	Up
[3]	LIF-R	Up
[4]	hK14	Up
[5]	AOC3	Down
[6]	GSAP	Down
[7]	NELL1	Down
[8]	GAMT	Down
[9]	CD164	Down
[10]	LGMN	Down
[11]	VPS37A	Down
[12]	VAMP5	Down
[13]	NFKBIE	Down
[14]	TMSB10	Down
[15]	PRKCQ	Down
[16]	PRDX1	Down
[17]	CASP-3	Down
[18]	CETN2	Down
[19]	LYN	Down

FIGURE 3 Alzheimer's disease (AD) classification based on the plasma ATN biomarkers and the 19-protein biomarker panel. A, Receiver operating characteristic (ROC) curves showing the AD classification results based on the plasma levels of candidate protein biomarkers. The classification results of the models integrating the plasma ATN biomarkers (i.e., plasma amyloid-beta [Aβ]$_{42/40}$ ratio, plasma tau level, and plasma neurofilament light polypeptide [NfL] level; yellow), the 19-protein biomarker panel (blue), and the plasma ATN biomarkers plus the 19-protein biomarker panel (red) in the Hong Kong Chinese AD discovery cohort. B, Bar chart showing the areas under the ROC curves (AUCs) according to the three AD classification models in the Hong Kong Chinese AD discovery cohort (AUC = 0.8735, 0.9816, and 0.9891 for ATN, 19 proteins, and ATN + 19 proteins, respectively). Data are mean ± standard error of the mean (ATN vs. 19 proteins: Z = 3.653, ATN vs. ATN + 19 proteins: Z = 3.991). C, Distribution of AD classification scores stratified by phenotype (n = 71 healthy controls [HCs], n = 101 patients with AD). The AD severity levels were designated according to the distribution of AD classification scores (normal, <0.3; mild, 0.3–0.8; severe, > 0.8). D–G, Associations between individual designated AD severity levels and AD-associated endophenotypes in the Hong Kong Chinese AD discovery cohort. Data are presented as box-and-whisker plots including maximum, 75th percentile, median, 25th percentile, and minimum values; plus signs (+) denote the corresponding mean values. D, Associations between individual cognitive performance indicated by Montreal Cognitive Assessment (MoCA) score and designated AD severity levels (n = 64, 17, and 91 for normal, mild, and severe levels, respectively; T = −2.396, −16.92, and −7.119 for normal vs. mild, normal vs. severe, and mild vs. severe, respectively). E, F, Associations between designated AD severity levels and brain volumetric data (n = 50, 12, and 47 for normal, mild, and severe levels, respectively). E, Hippocampal volume comparison (T = −2.397, −7.714, and −2.310 for normal vs. mild, normal vs. severe, and mild vs. severe, respectively). F, Gray matter volume comparison (T = −5.110 for normal vs. severe). G, Association between white blood cell counts and designated AD severity levels (n = 42, 8, and 51 for normal, mild, and severe levels, respectively; T = 2.734 for normal vs. severe; *P < 0.05, **P < 0.01, ***P < 0.001)

P < 0.05; Figure 4H). Moreover, we again found that the AD classification scores generated by the model were correlated with cognitive performance (Figure 4I, J). Therefore, these findings confirm that our integrative model accurately reflects AD-associated endophenotypes. Finally, combined analysis of the two independent cohorts (n = 142 patients with AD, n = 135 HCs; Table S1) revealed that our integrative model consistently and accurately classified AD with 90.51% sensitivity and 90.91% specificity (AUC = 0.9594; Figure S7A in supporting information). The resultant AD classification scores and designated AD severity levels also distinguished patients with AD from HCs and accu-

rately reflected cognitive decline (Figure S7B, C). Hence, these findings collectively demonstrate that our integrative model based on the 19-protein biomarker panel can classify AD and associated endophenotypes.

3.5 | The 19-protein biomarker panel accurately classifies AD with tau pathology

Compared to plasma ATN biomarkers, plasma p-tau181 has emerged as a more accurate and specific blood biomarker of AD[6] that indicates
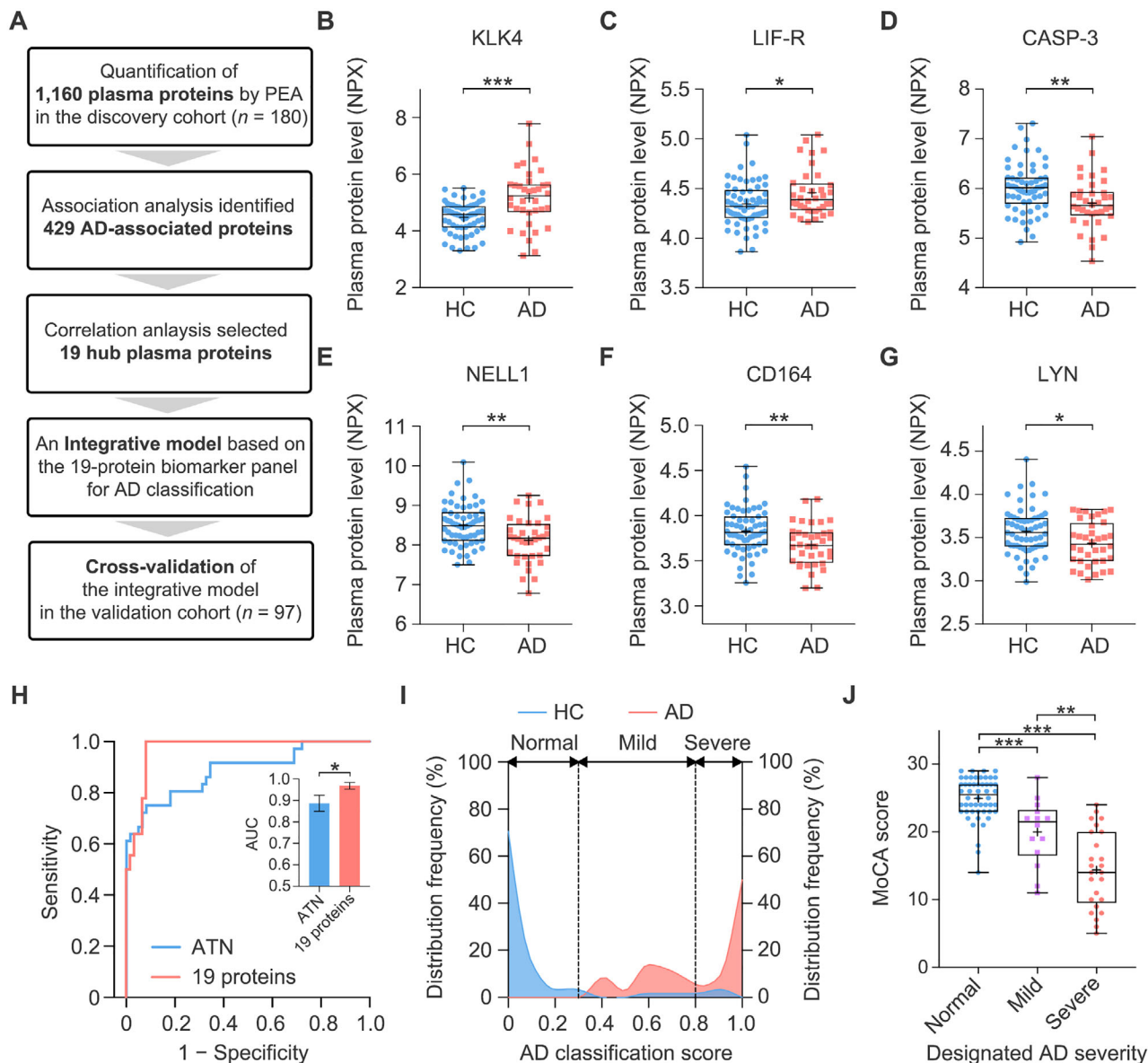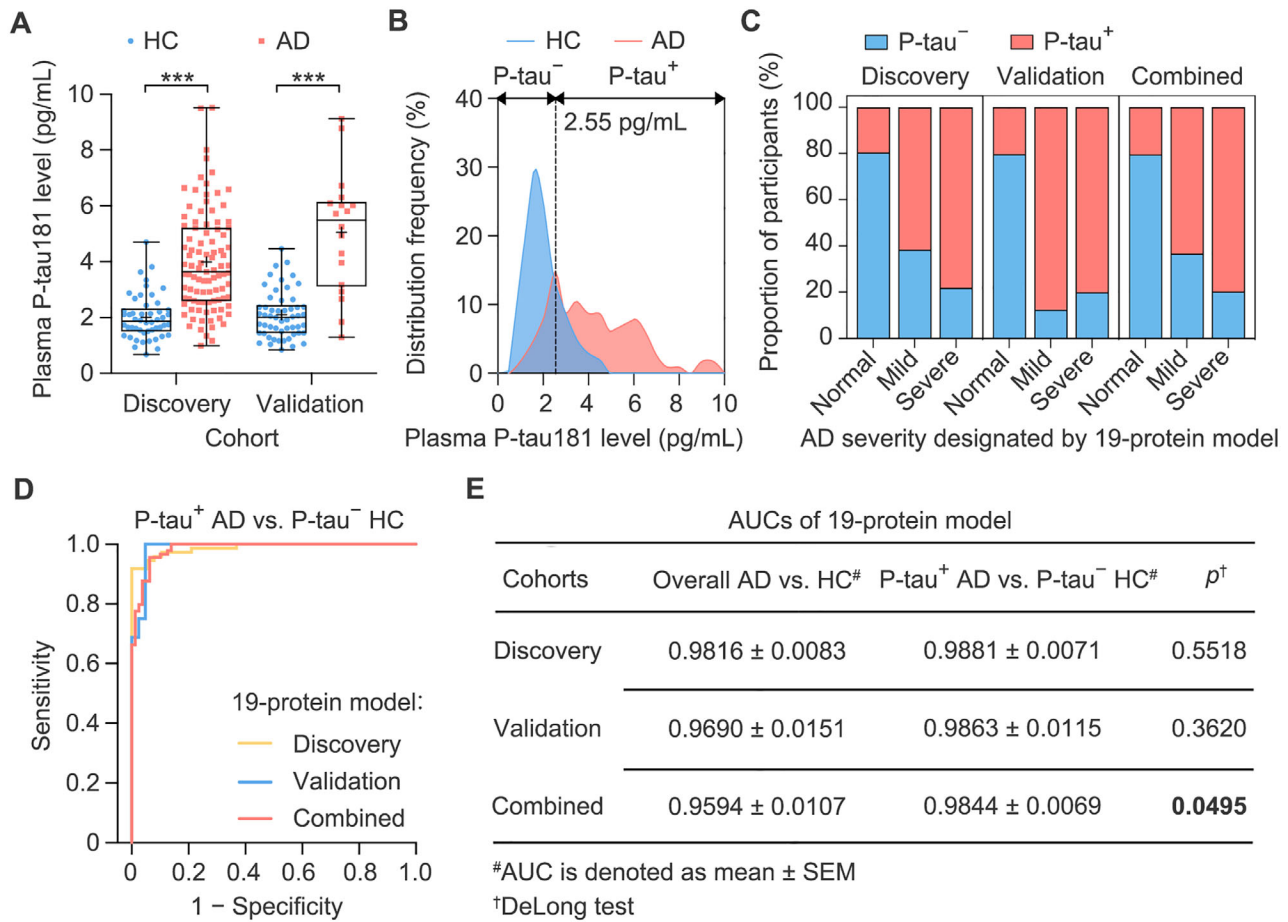
FIGURE 4 Validation of the 19-protein biomarker panel in an independent cohort. A, Pipeline for the establishment and evaluation of the integrative model based on the 19-protein biomarker panel. B–G, Individual plasma levels of KLK4 (B), LIF-R (C), CASP-3 (D), NELL1 (E), CD164 (F), and LYN (G) stratified according to Alzheimer's disease (AD) phenotype in the Hong Kong Chinese AD validation cohort. Data are presented as box-and-whisker plots including maximum, 75th percentile, median, 25th percentile, and minimum values; plus signs (+) denote corresponding mean values ($n = 61$ healthy controls [HCs], $n = 36$ patients with AD; $T = 4.315, 2.296, −2.887, −3.383, −2.983$, and $−2.501$ for KLK4, LIF-R, CASP-3, NELL1, CD164, and LYN, respectively). H, Receiver operating characteristic (ROC) curves and corresponding areas under the curve (AUCs) showing the performance of the models integrating the plasma ATN biomarkers (blue, AUC = 0.8871) and the 19-protein biomarker panel (red, AUC = 0.9690) for AD classification in the Hong Kong Chinese AD validation cohort. Data are mean ± standard error of the mean ($Z = 2.034$). I, Distribution of AD classification scores stratified by phenotype ($n = 61$ HCs, $n = 36$ patients with AD in the Hong Kong Chinese AD validation cohort). Three designated AD severity levels (normal, <0.3; mild, 0.3–0.8; severe, > 0.8) are indicated. J, Association between the individual Montreal Cognitive Assessment (MoCA) scores and designated AD severity levels ($n = 52, 14$, and 25 for normal, mild, and severe, respectively; $T = −4.621, −10.46$, and $−3.085$ for normal vs. mild, normal vs. severe, and mild vs. severe, respectively; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

the progression of tau pathology in the brain.[6,10] Consistently, in both of our independent cohorts, the plasma p-tau181 level was elevated in patients with AD (Figure 5A) and could classify AD and associated endophenotypes more accurately than the plasma ATN biomarkers (Figure S8 in supporting information). Therefore, we subsequently investigated the performance of our 19-protein model

to predict p-tau status using plasma p-tau181 as an indicator of tau pathology. We stratified our cohorts into p-tau181–negative (p-tau−) or p-tau181–positive (p-tau+) groups according to plasma p-tau181 level with a cutoff of 2.55 pg/mL derived from Youden's index (see Section 2.6; Figure 5B and Figure S9 in supporting information). The AD severity levels determined by the 19-protein model were

FIGURE 5 Performance of the 19-protein biomarker panel for classifying Alzheimer's disease (AD) with tau pathology. A, Individual plasma P-tau181 levels stratified by AD phenotype in the Hong Kong Chinese AD discovery and validation cohorts (discovery cohort: $n = 50$ healthy controls [HCs], $n = 97$ patients with AD; validation cohort: $n = 54$ HCs, $n = 18$ patients with AD; $T = 7.412$ and 8.431 for the tests in discovery and validation cohort, respectively). B, Distribution of plasma phosphorylated tau (p-tau)181 levels stratified by AD phenotype ($n = 104$ HCs, $n = 115$ patients with AD in the Hong Kong Chinese AD combined cohort). Individuals with plasma p-tau181 levels ≤ 2.55 or > 2.55 pg/mL were classified as p-tau181–negative (p-tau$^-$) and p-tau181–positive (p-tau$^+$), respectively. C, Proportions of p-tau$^-$ (blue) and p-tau$^+$ (red) individuals stratified by AD severity levels determined by the 19-protein model in the discovery (left), validation (middle), and combined (right) cohorts. D, Receiver operating characteristic (ROC) curves showing the performance of the 19-protein biomarker panel for AD classification in the p-tau181–stratified Hong Kong Chinese AD discovery (yellow), validation (blue), and combined (red) cohorts. E, Areas under the ROC curves (AUCs) of the 19-protein model in the Hong Kong Chinese AD discovery, validation, and combined overall cohorts as well as p-tau181–stratified cohorts

significantly correlated with p-tau status: ≈80% of individuals in the severe group were p-tau+ compared to ≈20% in the normal group (Figure 5C and Figure S10 in supporting information). Moreover, as p-tau status helped classify AD more accurately in terms of cognitive performance, brain region volumes, and plasma biomarkers (Figure S11 in supporting information), when we compared the plasma proteome specifically between p-tau+ AD patients and p-tau− HCs, we observed more prominent alterations of the 19 plasma hub proteins in p-tau+ AD in both the discovery and validation cohorts (Figure S6, S12; Table S7, S8; and Table A6 in supporting information). Concordantly, the 19-protein model accurately distinguished p-tau+ AD patients from p-tau− HCs in the discovery (AUC = 0.9881), validation (AUC = 0.9863), and combined cohorts (AUC = 0.9844; Figure 5D); notably, it differentiated between p-tau+ AD patients and p-tau− HCs significantly better than between AD patients and HCs in the overall cohorts (Figure 5E). Therefore, these results collectively demonstrate

that our 19-protein model has particularly high accuracy for classifying AD with tau pathology, providing the basis for developing a highly specific blood-based diagnostic tool for AD.

3.6 | Stage-dependent dysregulation of the plasma hub proteins in AD

The progression of AD can be marked by AD-associated endophenotypes (e.g., cognitive performance) and biomarker levels (e.g., plasma p-tau181).[1,2,10,25] However, the stages of AD lack clear biological definitions because of the lack of stage-specific biomarkers. Our 19-protein panel contains 10 plasma hub proteins that are correlated with plasma p-tau181 levels—7 of which are also correlated with cognitive (i.e., MoCA) scores (Tables S6, S9 in supporting information). Interestingly, further examination of how these plasma hub proteins

are dysregulated upon cognitive decline and the development of tau pathology revealed three types of changes (Figure 6 and Figure S13 in supporting information). First, three hub proteins—NELL1 and hK14 (expressed in the brain) and CETN2 (expressed in the brain and peripheral system)—were dysregulated in the early stages of AD (i.e., MoCA score > 25, plasma p-tau181 level < 2.5 pg/mL) and continued to change throughout the disease's progression. Second, the plasma levels of LYN, PRKCQ, and LIF-R were only altered in the early and/or intermediate stages (MoCA score: 10–25) and were relatively constant in the late stage. Third, KLK4, which is expressed by peripheral macrophages (Table S4), was only dysregulated in the late stage of AD when individuals have severe cognitive deficits (i.e., MoCA score < 15) and tau pathology (plasma p-tau181 level > 4 pg/mL). This suggests that the changes of certain plasma proteins (or biological processes) are associated with specific stages of AD. Determining the changes in the levels of these plasma hub proteins can indicate the status of corresponding biological processes in the blood of individuals, enabling inference of their AD stage. Therefore, these results demonstrate that the plasma hub proteins identified herein not only accurately distinguish patients with AD from HCs but more importantly can serve as a scale to provide biological annotations for AD staging.

4 | DISCUSSION

As blood-based tests are widely used to aid the diagnosis of metabolic, immune, or cardiovascular diseases,[45–47] the development of blood biomarkers for AD is a potential solution for both initial disease risk screening and long-term disease monitoring. Accordingly, in this study, we identified 19 AD-associated plasma hub proteins and developed a biomarker panel to classify AD and determine the disease severity of patients with AD. We systematically investigated the plasma proteome of AD by profiling 1160 plasma proteins using PEA, which is a high-throughput immunoassay technique that covers a broad concentration range with high precision and requires only minimal sample input (< 20 μL plasma). Our findings not only replicate known AD-associated plasma proteins such as IGFBP2, PPY, and VCAM1[12,13,15,16] but also reveal hundreds of novel proteins that are altered in the blood in AD. Subsequent correlation and network analyses classified these AD-associated plasma proteins into 19 protein clusters, each of which is involved in distinct biological pathways and can be represented by a single hub protein. We subsequently developed a blood-based biomarker panel based on these 19 AD-associated hub proteins to capture the overall plasma proteomic profile of patients with AD. Accordingly, we used this panel to establish a highly accurate model for the classification of AD and determination of disease severity.

Emerging evidence of ethnic differences in the blood proteome[48–50] strongly warrants evaluating the performance of known and newly identified AD blood biomarkers across ethnic groups. Accordingly, we comprehensively compared our findings in Chinese patients with AD with the findings of AD blood proteomics studies from populations of European descent. First, we showed that the changes in the plasma ATN biomarkers (i.e., A$\beta_{42/40}$, tau, and NfL) and plasma p-tau181 level in AD are consistent across ethnic groups: their changes in patients

with AD in our Chinese cohorts are comparable to those in previous studies on populations of European descent (Figure S1 and Figure 5A).[10,32-34] Second, regarding other AD-associated plasma proteins, our meta-analysis of 14 pilot AD and MCI proteomics studies demonstrates that besides proteins that exhibit ethnic-specific dysregulation in AD (e.g., BTC and CXCL1), 56 of the 77 AD-associated plasma proteins are consistently altered in both the Chinese population and populations of European descent (Table A1). Specifically, comparing our findings to a previous PEA-based proteomics study that assayed a subset of 270 proteins in a Swedish MCI cohort[17] revealed strong positive correlations between the assayed proteins in both cohorts (Figure S3B, C). In addition, we found that several plasma proteins become dysregulated in the early stage of AD, as indicated by their alterations in both AD and MCI (Figure S3C) as well as correlations between their respective levels and AD-associated endophenotypes when individuals have relatively normal cognitive performance and plasma p-tau181 levels (Figure 6). Our integrative model, which includes some of these plasma proteins (e.g., NELL1, CETN2, hK14, LYN, PRKCQ, and LIF-R), also distinguishes AD with mild cognitive deficits from HCs (Figure S5) and can reflect the degree of cognitive decline, brain atrophy, inflammation, and tau pathology (Figure 3D–G, 4J, 5C and Figure S7C, 10). Therefore, it is worth validating these protein candidates and our model in a future longitudinal study—the results of which might aid the development of a strategy for the early prediction and diagnosis of AD.

The concurrent use of multiple biomarkers to determine disease status, commonly termed "composite biomarker panels," is an effective method to fully exploit the predictive values of protein candidates. Such composite biomarker panels are widely used to predict cardiovascular diseases and aging.[21,51] In the present study, by conducting the most comprehensive characterization of the AD plasma proteome (to our knowledge) so far, we established a panel of 19 proteins that captures the overall proteomic profile of AD plasma and consequently classifies AD with high accuracy. Compared to existing blood biomarkers for AD classification, our 19-protein panel is significantly more accurate than the plasma ATN biomarkers (Figure 3A, 3B, 4H and Figure S7A) and comparably accurate to the plasma Aβ composite panel (AUC = 0.96), plasma p-tau181 (AUC = 0.85–0.98), and plasma p-tau217 (AUC = 0.96).[4,6,7,10] Moreover, emerging evidence shows that pathways related to inflammation, metabolism, and angiogenesis are also associated with AD.[52–54] Thus, our 19-protein panel captures a broader spectrum of peripheral biological processes compared to plasma A$\beta_{42/40}$, tau/p-tau, and NfL as biomarkers of Aβ pathology, tau pathology, and neurodegeneration, respectively. As recent studies imply the presence of subtypes of AD,[55–57] our protein panel can provide additional insights into the stratification of the disease, which might help identify potential subtypes and alternative pathological mechanisms of AD.

By investigating the changes in the plasma proteome in an AD cohort stratified according to sex, age, AD drug usage, and APOE ε4 genotype, we demonstrated that the levels of the 19 plasma hub proteins and plasma ATN biomarkers are consistently altered in AD independent of the abovementioned intrinsic and external factors (Figure S4 and Table A5). Therefore, these plasma proteins can be
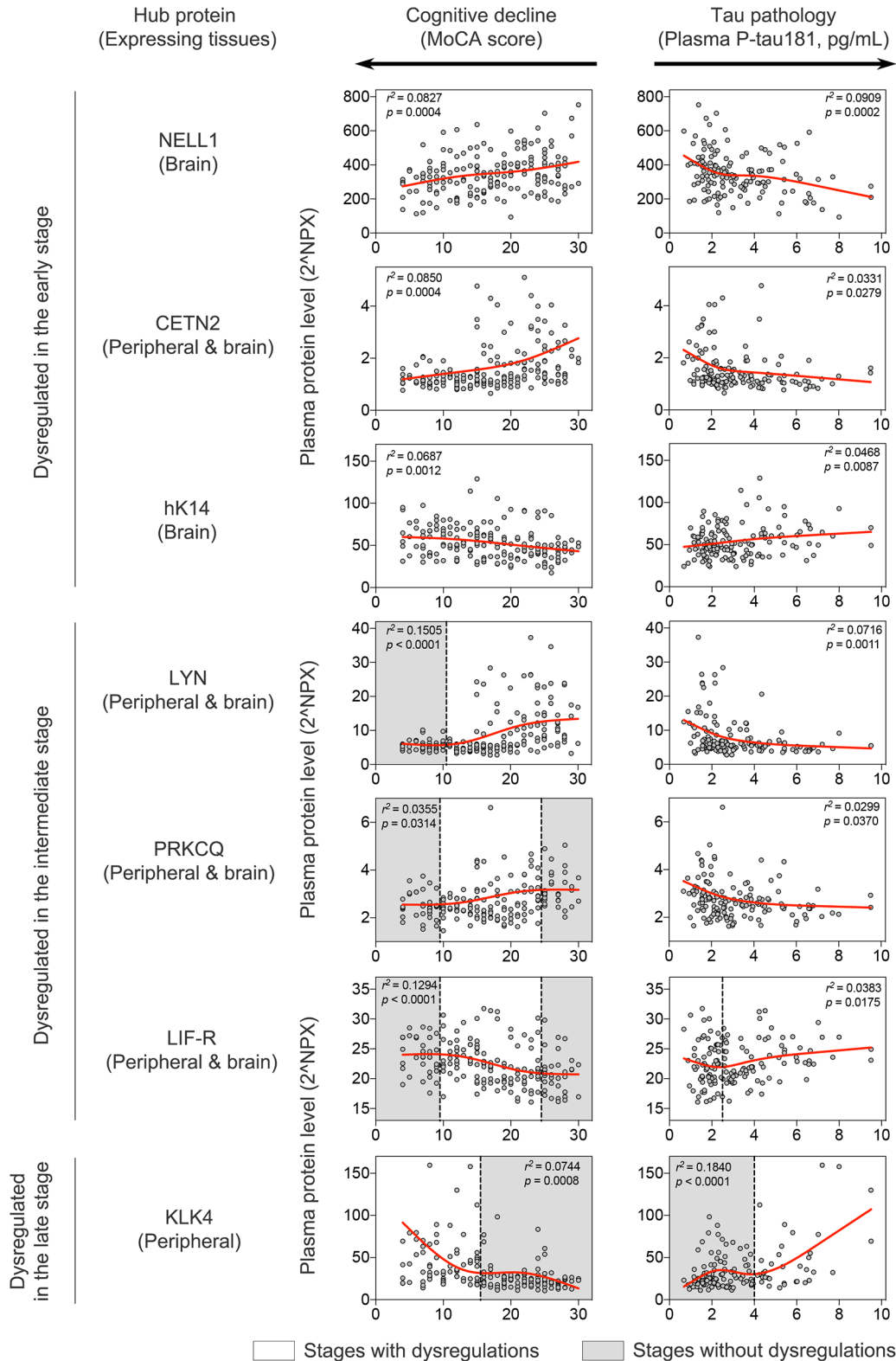
FIGURE 6 Alzheimer's disease (AD) stage-dependent dysregulation of seven plasma hub proteins. Correlations between the plasma levels of NELL1, CETN2, hK14, LYN, PRKCQ, LIF-R, and KLK4 and cognitive decline indicated by Montreal Cognitive Assessment (MoCA) scores (left) as well as tau pathology indicated by plasma phosphorylated tau (p-tau)181 level (right) in the Hong Kong Chinese AD discovery cohort. Red splines denote the locally weighted scatterplot smoothing (LOWESS) fit lines of corresponding proteins, and vertical dashed lines indicate the inflection points. r^2, Pearson's correlation coefficient; 2^NPX, linear form of normalized protein expression

developed into a biomarker panel that can classify AD in the general population. Integrating additional AD-associated genetic variants into the model, such as *APOE ε*4 genotype, might improve its performance. Of note, while sex, age, AD drug usage, and *APOE ε*4 genotype did not obviously affect the alteration of plasma proteins in AD, we did observe significant associations between these factors and the levels of some plasma proteins (e.g., significantly lower plasma A$\beta_{42/40}$ levels in females vs. males and in *APOE ε*4 carriers vs. noncarriers; Table A5), suggesting that these factors potentially modulate the basal levels of AD-associated plasma proteins. This is consistent with recent findings in populations of European descent showing that sex, age, and genetic components play essential roles in regulating the plasma proteome.[21,58] Therefore, it is of interest to validate such effects of sex, age, and *APOE ε*4 genotype on the AD plasma proteome in a larger dataset, which may expand our understanding of how they modulate disease risks and associated pathologies.[59,60]

Current definitions of AD stages are mainly based on an individual's behaviors and cognitive performance.[1,2,25] Although existing AD biomarkers such as plasma p-tau and NfL are good indicators of AD progression,[5–9] they might not clearly differentiate disease stages given their relatively constant alteration throughout the disease course (Figure S8C–H).[5,7–10] In the present study, we identified seven plasma hub proteins and associated biological processes that exhibit AD stage-specific dysregulation in the blood. Two proteins secreted from the brain (i.e., NELL1 and hK14) start to be dysregulated in the earliest stage of AD (i.e., MoCA score > 25; plasma p-tau181 < 2.5 pg/mL); this is followed by the alteration of LYN and PRKCQ-associated peripheral immune response and apoptosis (i.e., MoCA score: 10–25; plasma p-tau181: 2.5–4 pg/mL), and finally KLK4-associated extracellular matrix disassembly and angiogenesis of endothelial cells (i.e., MoCA score < 15; plasma p-tau181 > 4 pg/mL; Figure 6 and Table S3). This presents clear separation of AD stages in terms of the changes of biological processes in the blood. Therefore, integrating these seven plasma hub proteins into the current AD progression indicators (e.g., MoCA score and plasma p-tau181 level) not only accurately classifies AD (AUC = 0.9751 for 7 proteins + p-tau181) but also provides stage-specific biological annotations to the disease, which helps delineate the disease stages. Future studies of the dysregulatory patterns of additional proteins might further specify and/or subdivide these AD stages. In addition, proteins or protein clusters that are dysregulated in the early and/or intermediate stages of AD might actually be involved in AD-causative pathways and not merely markers of cell or tissue dysregulation in AD. For example, LIF-R (leukemia inhibitory factor receptor) is dysregulated in the intermediate stage of AD (Figure 6) and is elevated in both the blood and brain in individuals with MCI or AD.[17,61,62] Moreover, VCAM1 (vascular cell adhesion molecule 1), which was coregulated with LIF-R in the same cluster in the present study, plays pathogenic roles in cognitive decline and hippocampal impairment in aging.[52,63] Therefore, future functional studies on this protein cluster might provide insights into the pathological mechanisms of AD. Similarly, considering that many proteins and protein clusters revealed in our study showed early dysregulations in AD (Figure 6 and Table S3), more in-depth characterization of these plasma proteins

will open new avenues for investigating the mechanisms of AD and developing intervention strategies.

Nevertheless, some issues must be addressed to develop the 19 proteins identified herein into a biomarker panel for clinical use. First, although we validated the changes in most of the 19 plasma hub proteins in an independent cohort (Figure 4 and Figure S6), PRDX1, VAMP5, and GAMT were inconsistently regulated in AD (Figure S6). Given that these three proteins were consistently dysregulated in p-tau$^+$ AD blood in the p-tau181–stratified discovery and validation cohorts (Figure S12) and are reported to be involved in AD[64,65] and aging,[66] the above inconsistencies are likely due to the small sample size of our validation cohort. Therefore, replication studies examining the 19-protein biomarker panel in a larger population are required to clarify how these proteins are regulated in AD. Second, despite the high performance of our 19-protein biomarker panel in predicting AD-specific tau pathology (Figure 5C–E) as well as the fact the levels of at least two proteins in our panel—CASP-3 and LIF-R—are unaltered in Parkinson's disease and progressive supranuclear palsy,[17] the development of a highly sensitive and specific blood-based test for AD requires additional testing of the model on other neurodegenerative diseases. Finally, given that the transcriptome and epigenome are reported to be altered in the blood in AD,[67,68] it is worthwhile to investigate the interactions among the AD-associated transcriptome, epigenome, and proteome, which will unveil the pathological roles of the peripheral system in AD and help develop a more comprehensive blood-based test.

In conclusion, we systematically studied the plasma proteome of patients with AD, identified an AD biomarker panel comprising 19 plasma proteins, and established a highly accurate integrative model for AD classification and disease staging. Our findings not only serve as a foundation for the development of a high-performance, blood-based test for AD screening and monitoring in clinical settings but also provide abundant protein targets for future therapeutic development.

CONFLICTS OF INTEREST

H.Z. has served on scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, and CogRx; has given lectures in symposia sponsored by Fujirebio, AlzeCure, and Biogen; and is a cofounder of Brain Biomarker Solutions (BBS) of Gothenburg AB, which is part of the GU Ventures Incubator Program (outside submitted work). All other authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Y.J., X.Z., A.K.F., and N.Y.I. conceived of the study; F.C.I., P.C., K.C., R.M.L., E.P.T., B.W.W., A.L.C., V.C.M., and T.C.K. organized patient recruitment and sample collection; Y.J. and N.C.L. performed the experiments; Y.J. and X.Z. set up the data-processing pipelines; Y.J., X.Z., N.C.L., Y.C., K.Y.M., J.H., H.Z., A.K.F., and N.Y.I. analyzed the data; and Y.J., X.Z., H.Z., A.K.F., and N.Y.I. wrote the manuscript with input from all authors.

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SUPPORTING INFORMATION

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