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Novel plasma protein biomarkers: A time-dependent predictive model for Alzheimer's disease

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HIGHLIGHTS

- CSF collection via lumbar puncture is invasive, difficult to implement widely.
- Blood tests are minimally invasive, cost-effective and accessible, suitable for large-scale use in primary care.
- Prior studies viewed Alzheimer's disease as binary, ignoring its protracted course from pathological changes to onset, focusing on protein signatures between two groups.
- Our study is the first to predict Alzheimer's disease risk dynamically using novel plasma protein biomarkers.

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ABSTRACT

Background: The accurate prediction of Alzheimer's disease (AD) is crucial for the efficient management of its progression. The objective of this research was to construct a new risk predictive model utilizing novel plasma protein biomarkers for predicting AD incidence in the future and analyze their potential biological correlation with AD incidence.
Methods: A cohort of 440 participants aged 60 years and older from the Alzheimer's Disease Neuroimaging Initiative (ADNI) longitudinal cohort was utilized. The baseline plasma proteomics data was employed to conduct Cox regression, LASSO regression, and cross-validation to identify plasma protein signatures predictive of AD risk. Subsequently, a multivariable Cox proportional hazards model based on these signatures was constructed. The performance of the risk prediction model was evaluated using time-dependent receiver operating characteristic (t-ROC) curves and Kaplan-Meier curves. Additionally, we analyzed the correlations between protein signature expression in plasma and predicted AD risk, the time of AD onset, the expression of protein signatures in cerebrospinal fluid (CSF), the expression of CSF and plasma biomarkers, and APOE $\varepsilon 4$ genotypes. Colocalization and Mendelian randomization analyses was conducted to investigate the association between protein features and AD risk. GEO database was utilized to analyze the differential expression of protein features in the blood and brain of AD patients.
Results: We identified seven protein signatures (APOE, CGA, CRP, CCL26, CCL20, NRCAM, and PYY) that independently predicted AD incidence in the future. The risk prediction model demonstrated area under the ROC curve (AUC) values of 0.77, 0.76, and 0.77 for predicting AD incidence at 4, 6, and 8 years, respectively. Furthermore, the model remained stable in the range of the 3rd to the 12th year (ROC $\geq$ 0.74). The low-risk group, as defined by the model, exhibited a significantly later AD onset compared to the high-risk group ($P < 0.0001$). Moreover, all protein signatures exhibited significant correlations with AD risk ($P < 0.001$) and the time of AD onset ($P < 0.01$). There was no strong correlation between the protein expression levels in plasma and CSF, as well as AD CSF biomarkers. APOE, CGA, and CRP exhibited significantly lower expression levels in APOE $\varepsilon 4$ positive individuals ($P < 0.05$). Additionally, colocalization analysis reveals a significant association between AD and SNP loci in APOE. Mendelian randomization analysis shows a negative correlation between NRCAM and AD

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risk. Transcriptomic analysis indicates a significant downregulation of NRCAM and PYY in the peripheral blood of AD patients (P < 0.01), while APOE, CGA, and NRCAM are significantly downregulated in the brains of AD patients (P < 0.0001).

Conclusion: Our research has successfully identified protein signatures in plasma as potential risk biomarkers that can independently predict AD onset in the future. Notably, this risk prediction model has demonstrated commendable predictive performance and stability over time. These findings underscore the promising utility of plasma protein signatures in dynamically predicting the risk of AD, thereby facilitating early screening and intervention strategies.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a gradual decline in cognitive function and the presence of psychiatric disturbances (McDade, 2022). Despite continuous research, the underlying mechanisms of AD remain incompletely elucidated (Tatulian, 2022). Presently available treatment modalities merely alleviate symptoms and do not impede disease progression (Ogbodo et al., 2022). Nevertheless, it is established that the development of AD occurs years prior to the manifestation of overt symptoms (Guzman-Martinez et al., 2021). Early identification of individuals at high risk for developing AD through the utilization of biomarker-based predictive models, following interventions targeting potential risk factors, holds promising potential to control the incidence of the disease (Zhang et al., 2021, Graff-Radford et al., 2021, van Oostveen & de Lange, 2021).

Biomarkers play a crucial role in assessing an individual's susceptibility to future disease risk and predicting the time of AD onset (Mantzavinos and Alexiou, 2017, Klyucherev et al., 2022). The National Institute on Aging-Alzheimer's Association (NIA-AA) proposed the "ATN" framework in 2018 (Jack et al., 2018). This framework defines and investigates AD at the biomarker level, utilizing cerebrospinal fluid (CSF) and positron emission tomography (PET) biomarkers, including β-amyloid 42 (Aβ42), total tau (t-tau), and phosphorylated tau (p-tau). However, the current methods employed for the collection of CSF via lumbar puncture are invasive and not feasible for widespread implementation (Simrén, Elmgren, Blennow and Zetterberg, 2023). The synthesis of PET radiotracers necessitates specialized equipment such as cyclotrons, which are limited in global availability (Delaby et al., 2022). Additionally, these techniques are costly, involve radioactivity, and demand highly skilled personnel, thereby increasing operational thresholds (Schneider, Hampel and Buerger, 2009). Consequently, there is a necessity to develop novel biomarkers that are more suitable for extensive application in primary healthcare facilities to enable early prediction of AD risk (Awasthi, Spellman, & Hatcher, 2022).

In comparison to PET and CSF, blood testing offers several advantages that render it an ideal option for developing biomarkers for AD (Henriksen et al., 2014). The minimally invasive nature, cost-effectiveness, and widespread accessibility of blood testing make them particularly well-suited for large-scale implementation within primary healthcare facilities (Teunissen et al., 2022). Additionally, there exists evidence indicating that the components in blood undergo frequent exchanges with the brain, and preclinical pathological changes in AD can be observed in blood samples (Banack, Stark and Cox, 2022, Elahi et al., 2020, Ashton et al., 2021, Zetterberg and Burnham, 2019). Previous research has shown promise in utilizing plasma biomarkers to predict AD risk. For example, tau proteins, in combination with other measurable indicators, have proven effective in predicting AD onset after a span of four years (Palmqvist et al., 2021). Machine learning algorithms that utilize a set of plasma proteins have demonstrated the capability to predict the progression of AD from the prodromal stage up to four years in advance (Araújo et al., 2022) or predict the transition from mild cognitive impairment (MCI) to AD within a timeframe of three years (Kononikhin et al., 2022).

However, it is important to note that previous research has predominantly concentrated on framing AD onset as a binary classification task, wherein participants are categorized into two groups based on the occurrence of AD at a specific time point, and differential protein signatures between these groups are identified. However, in reality, the progression from pathological changes to AD onset encompasses a protracted disease course (Yuyama et al., 2022). Consequently, there is a necessity to elucidate the risk of AD and dynamically predict the time at which it occurs (Long et al., 2022, Sharma, Anand, Badr and Qiu, 2021, Vromen et al., 2022). It requires selecting and modeling signatures with sustained effects on the onset of AD, in order to predict the disease progression over several consecutive years rather than predicting at a single specific point. It would be crucial to develop time-dependent risk biomarkers in plasma, as they can additionally provide insights into the mechanisms of AD as well as identify potential therapeutic targets (Elahi et al., 2020). Such biomarkers would increase the potential clinical applicability of the risk predictive model and contribute further research value to the potential plasma biomarkers.

In this research, we utilized proteomics and longitudinal data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database. Cox regression, LASSO regression, and cross-validation were employed to identify plasma protein signatures and construct a multi-factor Cox proportional hazard regression model for AD risk prediction. A multidimensional evaluation and analysis of the risk prediction model and the protein signatures have also been conducted. To the best of our knowledge, this research is the first to develop a method for dynamically predicting AD risk using novel plasma protein biomarkers. This innovative approach opens up new avenues for exploring the mechanisms of AD and potentially transforming into a clinical tool for AD risk assessment in the future.

2. Methods

2.1. Source of data

The participants in this research were sourced from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). ADNI aims to provide a systematic data platform for assessing and measuring the early transformation of AD through the integration of imaging, clinical neuropsychology, and fluid biomarkers (Petersen et al., 2010). Written informed consent was obtained from all participants for all examination procedures conducted in ADNI. The ADNI cohort is a dynamically recruited study group that includes participants who were already diagnosed with AD upon entry and those who were not diagnosed with AD, with long-term follow-up. AD is defined as a clinical presentation and diagnosis of dementia consistent with Alzheimer's disease, while participants not meeting this criterion are defined as non-AD. In our study, to develop an AD risk prediction model based on plasma proteins, we included non-AD participants who had not been diagnosed with AD at the time of entering the ADNI cohort and who were over 60 years old. Data collected at entry, defined as baseline data, includes demographic information, scale scores, and other biological markers. It is important to note that participants' diagnostic information is dynamically assessed, with AD diagnosis status re-evaluated and recorded every six months until diagnosed with AD or lost to follow-up. Baseline data collected for this study includes demographic information, APOE $\varepsilon 4$ genotypes, Mini-Mental State Examination (MMSE) scores,

Clinical Dementia Rating (CDR) cognitive assessment scores, cerebrospinal fluid (CSF) biomarkers, plasma biomarkers, and plasma proteomic measurements from the ADNI database. The expression level of plasma proteins of non-AD participants from plasma proteomic data is the primary predictor variable used to build the model. Plasma samples were collected at the time of participant enrollment in ADNI. In this study, plasma proteomic data is the primary predictor variable used to construct the model. The participants' plasma samples were collected at the time of their enrollment in the ADNI cohort. The plasma proteomic was analyzed using the Luminex xMAP platform, will be the primary predictors in our AD risk prediction model. This data and processing methods are available in the "Biomarkers Consortium Plasma Proteomics Project RBM multiplex data" dataset within ADNI.

2.2. Identification of protein signatures and construction of the risk prediction model

Single-factor Cox regression was conducted with plasma protein levels as the independent variable and follow-up time and AD incidence as the dependent variables. Proteins that were significantly associated with the risk of AD development were preliminary selected ($P < 0.05$). Subsequently, LASSO regression was performed using the R package "glmnet" to further narrow down the range of candidate proteins. The lambda value was selected based on the one-standard-error criterion (lambda.1se) for protein signature screening to develop a more concise multi-factor Cox regression prediction model.

The multi-factor Cox regression prediction model was visualized as a forest plot using the "ggforest" function through the R package "survminer", displaying the Hazard Ratio (HR) and confidence interval of protein signatures.

A nomogram of the multi-factor Cox regression prediction model was constructed using the R package "rms" to visualize the calculated AD risk.

2.3. Performance validation of the predictive model

To assess the AD risk dynamic predictive performance of the predictive model, the participants were randomly divided into a train set and a test set in a 7:3 ratio. R package "time-ROC" was used to calculate the area under the time-dependent receiver operating characteristic (t-ROC) curve of the multi-factor Cox regression predictive model for the whole sample and the test set participants separately. Additionally, ROC curves for predicting AD risk were plotted using age, gender, education, APOE $\varepsilon 4$ carrier status, baseline MMSE score, CDR-SB score AD CSF biomarkers and AD plasma biomarkers compared with the predictive model.

The "surv_cutpoint" function from the R package "survminer" was used to calculate the cutoff value of the AD risk score generated by the model. The participants were then divided into high-risk and low-risk groups for developing AD using median as the cutoff value, and Kaplan-Meier analysis was performed to compare the time of AD onset between the two groups. T-test was performed to compare differences of AD incidence, age, gender, education years, APOE $\varepsilon 4$ carrier status, MMSE and CDR-SB scores, AD CSF biomarkers, plasma biomarkers and expression of APOE, CGA, CRP in CSF between high-risk and low-risk group.

2.4. Analysis of protein signatures

The R package "ggpubr" was used to create box plots to analyze the differential expression of protein signatures between the high-risk and low-risk groups for AD, as well as the relationship between the expression levels of these protein signatures and the APOE $\varepsilon 4$ genotype.

The R package "survminer" was used to calculate the cutoff values of protein signature expression levels, and Kaplan-Meier curves were used to analyze the relationship between the expression levels of each protein signature and the time of AD onset.

The R package "corrplot" was used for Pearson analysis to investigate the correlation between protein signatures expression in plasma, the correlation of protein signatures expression in plasma with CSF, and the correlation of protein signatures expression with AD CSF biomarkers Aβ, tau, and p-tau expression.

2.5. Colocalization and Mendelian randomization analysis of characteristic protein-coding genes

Bayesian colocalization analysis assesses the probability that two traits share the same causal variant. We used the "coloc" package with default parameters (https://github.com/chr1swallace/coloc). The ieugwasr_to_coloc function was employed to extract colocalization data, and the coloc.abf function was used to perform genetic colocalization analysis on two potentially related phenotypes to determine whether they share the same causal genetic variation within the eQTL genetic distance region of the corresponding genes. A colocalization evidence threshold was defined as PP.H4.abf > 0.8, and we used the locuscompare function from the locuscompare package and the stack_assoc_plot function from the gassocplot2 package for visualization.

We downloaded the AD GWAS dataset "ieu-b-5067" from the IEU Open GWAS Project (https://gwas.mrcieu.ac.uk/), which includes 954 cases, 487,331 controls, and 12,321,875 SNPs. The eQTL data for the seven coding genes of protein signatures (where coding gene of CGA is CHGA, and the others share names with their respective proteins) was sourced from the eQTLGen Consortium (https://eqtlgen.org/). We then conducted Bayesian colocalization analysis between these characteristic genes and the AD GWAS dataset, where only APOE, CHGA, and NRCAM had enough SNPs for further analysis.

Mendelian Randomization (MR) analysis was conducted to assess the causal relationship between coding genes of protein signatures and AD. We screened for statistically significant single nucleotide polymorphisms (SNPs) as instrumental variables (IVs) for subsequent studies. After extracting the relevant data, we calculated the proportion of variance explained (R2) and the F statistic using the formula $R^2 = (2 \times \text{eaf} \times (1 - \text{eaf}) \times \text{beta}^2)$, $F = R^2 \times \frac{(N-2)}{(1-R^2)}$. IVs were selected under the following conditions: $P < 1e\text{-}05$, linkage disequilibrium parameter $R^2 < 0.001$, and genetic distances > 10,000 kb, ensuring the independence of IVs. MR Egger, Weighted Median, Inverse Variance Weighted (IVW), Simple Mode, and Weighted Mode were the five methods used to test the causal relationship between the gene signatures and AD. The GWAS data used for exposure and outcome was the same as in the colocalization analysis. Only NRCAM had a sufficient number of significant SNPs. Heterogeneity was considered present if the significance value of Cochran's Q statistic was less than 0.05. A leave-one-out study was performed to determine how eliminating one genetic variant from the MR analysis affected the results. Funnel plots were used to test for bias in the results.

2.6. Expression analysis of characteristic protein-coding genes

We downloaded two datasets, GSE140829 and GSE122063, from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The former contains peripheral blood samples from 204 AD patients and 249 controls, while the latter contains brain tissue samples from 56 AD patients and 44 controls. The downloaded data was in MINiML format and included complete data from two GSE platform samples. The extracted data were normalized using log2 transformation. Based on platform annotation information, probe IDs were converted to gene symbols, and probes mapping to multiple genes were excluded. For probes mapping to the same gene, the mean expression value was calculated. We used the Wilcoxon rank-sum test to examine differences in the expression of specific genes between AD patients and controls, with $P < 0.05$ considered statistically significant. Boxplots were generated using the R

package boxplot for visualization.

3. Results

3.1. Summary of research participants

A total of 440 non-AD participants who underwent plasma proteomics at baseline from the ADNI database were included in our research. During the follow-up period, 216 participants whose diagnosis changed from non-AD to AD are defined as "Developing AD," while the 224 participants who did not develop AD by the last follow-up are categorized as non-AD. The baseline characteristics of the research participants, including age, gender, education level, APOE ε4 carrier status, MMSE scores, CDR-SB scores, CSF biomarkers (CSF-Aβ, CSF-tau, CSF-p-tau), plasma biomarkers (Aβ42, Aβ40, NfL), onset time or follow-up time are presented in Table 1.

3.2. Identification of risk prediction protein signatures

Single-factor Cox regression analysis screened 23 candidate proteins significantly associated with the risk of AD ($P < 0.05$) for further analysis. Using LASSO regression and cross-validation, 7 independent protein signatures that can predict the risk of AD were identified (Fig. 1A and B), including apolipoprotein E (APOE), chromogranin A (CGA), C-reactive protein (CRP), chemokine ligand 26 (CCL26), chemokine ligand 20 (CCL20), neural cell adhesion molecule (NRCAM), and peptide tyrosine tyrosine (PYY).

As shown in Fig. 1C, APOE, CGA, CRP, CCL20, and NRCAM were considered protective factors for AD incidence ($HR < 1$), while CCL26 and PYY were considered risk factors for AD incidence ($HR > 1$). Utilizing these protein signatures, risk scores for developing AD can be calculated using the parameters in Table 2.

3.3. Nomogram and Validation of the Risk Predictive Model

The nomogram (Fig. 2) depicts a column chart of the risk predictive model based on protein signatures. By inputting the expression of protein signatures, corresponding scores can be calculated to quantify the risk of future AD onset.

Time-dependent ROC curves were then used to evaluate the model's classification ability for AD risk prediction through sensitivity and specificity. The participants were divided into a train set and a test set in a 7:3 ratio. Fig. 3A illustrates the area under the ROC curve (AUC) for AD onset prediction across each year from the first to the twelfth year of follow-up, for both the total sample and the test set. Overall, the Cox model demonstrates good fitting, with consistent predictive performance from the 3rd to the 12th year. For example, at the 4th, 6th, and 8th years, Fig. 3B and C present the ROC curves for the total sample and the test set, respectively. These highlight that the Cox model maintains strong and stable predictive performance over the observed period, indicating its robustness in predicting AD onset across different time points. The risk score, calculated based on the protein signatures, exhibited a higher AUC compared to APOE ε4 carrier status, CSF biomarkers and plasma biomarkers in both the overall sample (Fig. 3D) and the test set (Fig. 3E), indicating that the model had superior performance to these individual biomarkers in the risk prediction of AD. By utilizing the median of the risk scores as the threshold, the participants were divided into low-risk and high-risk groups for AD, and Kaplan-Meier analysis was performed. As shown in Fig. 3F, over time, the AD incidence in the low-risk group was significantly lower than that in the high-risk group ($P < 0.0001$). Additionally, there are significant differences between the low-risk and high-risk groups in terms of AD onset rate, gender, APOE ε4 carrier status, MMSE scores, CDR-SB scores, CSF-Aβ, and CSF-CGA (Table 3, $P < 0.05$).

Table 1
Baseline characteristics of all participants.

Characteristics	Developing AD	Not developing AD	Total
N	216	224	440
Age, mean (SD), years	74.5±7.1	75.0±7.2	74.8±7.1
Male gender, n (%)	137(63.4%)	137(61.2%)	276 (62.7%)
Education years, mean (SD), years	15.8±2.8	15.4±3.1	15.6±3.0
APOE ε4 carriers, n (%)	136(63.0%)	76(33.9%)	212 (48.2%)
MMSE scores, mean (SD)	26.8±1.7	27.7±1.8	27.3±1.8
CDR-SB scores, mean (SD)	1.8±0.9	1.0±0.9	1.4±0.97
CSF-Aβ, mean (SD), pg/mL	357.1±423.8	691.5±693.6	527.3 ±600.3
CSF-tau, mean (SD), pg/mL	166.6±184.8	145.2±149.9	155.7 ±168.1
CSF-p-tau, mean (SD), pg/mL	16.7±19.1	13.6±15.1	15.1±17.2
Aβ42, mean (SD), pg/mL	35.40±12.9	36.7±11.9	36.0±12.5
Aβ40, mean (SD), pg/mL	148.7±59.6	150.4±49.2	149.5 ±54.7
NfL, mean (SD), pg/mL	42.9±28.1	39.1±29.4	40.8±28.9
Onset time / Follow-up time, mean (SD), months	33.3±29.4	59.3±49.9	46.5±43.2

Abbreviations: APOE ε4, Apolipoprotein E ε4 allele; MMSE, mini-mental state examination; CDR-SB, Clinical Dementia Rating Scale-Sum of Boxes; Onset time is defined as the duration from the time participants in the "Developing AD" group enter the cohort until they are first diagnosed with AD. Follow-up time is defined as the duration from the time participants in the "Not developing AD" group enter the cohort until their last follow-up visit."

3.4. Protein Signatures Validation and Analysis

To further explore the validation and analysis of the correlation between protein signatures and AD, we conducted an assessment of the differential expression of these protein signatures between the high-risk and low-risk AD groups (Fig. 4). Significant disparities in expression levels were observed for all protein signatures ($P < 0.001$). Specifically, CCL26 and PYY demonstrated elevated expression levels in the high-risk group, while APOE, CGA, CRP, CCL20, and NRCAM exhibited higher expression levels in the low-risk group.

The ability of individual protein signatures to predict the time of AD onset was also assessed. Participants were divided into high expression and low expression groups for each protein, using the median expression level of each protein feature as a cutoff value. Kaplan-Meier analysis was then conducted (Fig. 5). The expression levels of these protein signatures are significantly associated with the incidence of AD. Specifically, higher expression levels of APOE ($P < 0.0001$), CGA ($P < 0.0001$), CRP ($P = 0.003$), CCL20 ($P < 0.01$), and NRCAM ($P < 0.01$) are associated with a lower risk of AD, while higher expression levels of CCL26 ($P < 0.01$) and PYY ($P < 0.01$) are associated with a higher risk of AD.

Furthermore, we analyzed the correlation between the expression levels of the protein signatures, their expression levels in blood and CSF, their correlation with AD CSF biomarkers, and their correlation with the APOE ε4 genotype. The expression levels of these proteins in plasma did not exhibit significant correlations (Fig. 6A). With the exception of a positive correlation observed between plasma CRP and CSF CGA, the expression levels of APOE, CGA, and CRP in CSF (CSF-APOE, CSF-CGA, and CSF-CRP) did not demonstrate a strong correlation with the protein signature expression levels in plasma (Fig. 6B). Additionally, the expression levels of the protein signatures in plasma did not exhibit significant correlations with other AD CSF biomarkers, including Aβ, tau, and p-tau (Fig. 6C) and AD plasma biomarkers including Aβ42, Aβ40, NfL (Fig. 6D).

It is widely recognized that, from a genetic perspective, there exists a correlation between the APOE ε4 genotype and an elevated susceptibility to AD (Raulin et al., 2022). As illustrated in Fig. 7, individuals with a positive APOEε4 genotype exhibited significantly diminished ($P < 0.05$) expression levels of APOE, CGA, and CRP. However, the

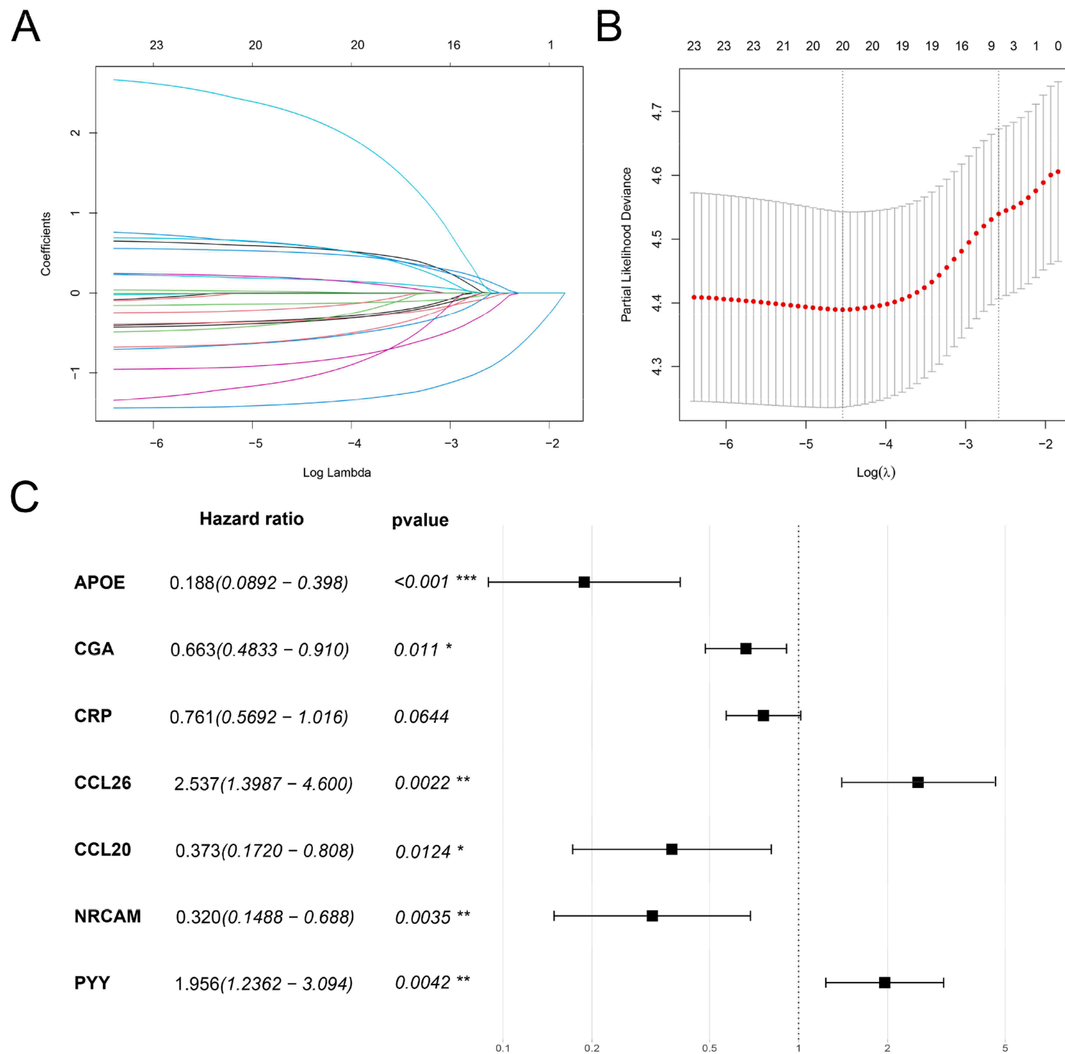

Fig. 1. Identification of protein signatures for AD risk. (A) Least absolute shrinkage and selection operator regression of the proteins. (B) Cross-validation for tuning the parameter selection in the LASSO regression. (C) Forest plot based on the Cox proportional hazard regression model indicated that APOE, CGA, CRP, CCL20, and NRCAM were protective factors for not developing AD while CCL26 and PYY were risk factors.

Table 2
The protein signatures and their coefficients. Based on the expression levels of these proteins in plasma, the AD onset risk score can be calculated using the following formula. Risk score = (APOE (ug/ml) *-0.919393936) + (CGA (ng/mL) *-0.050841006) + (CRP (ug/mL) *-0.008067947) + (CCL26 (pg/mL) *0.037093200) + (CCL20 (pg/mL) *- 0.004297646) + (NRCAM (ng/mL) *-0.211809013) + (PYY (pg/mL) *0.133760436).

No.	Gene name	Coefficients
1	APOE	-0.919393936
2	CGA	-0.050841006
3	CRP	-0.008067947
4	CCL26	0.037093200
5	CCL20	-0.004297646
6	NRCAM	- 0.211809013
7	PYY	0.133760436

expression levels of CCL26, CCL20, NRCAM, and PYY were found to be unrelated to the APOE$\varepsilon 4$ genotype ($P > 0.05$).

3.5. External Data Analysis of Characteristic Proteins

We further analyzed the seven protein signatures using external data. Colocalization analysis indicated that the SNPs of APOE were significantly associated with AD and driven by the same causal mutation site (PP.H4.abf > 0.8). This finding demonstrated a close genetic correlation between AD and APOE, which is consistent with previous studies and our own (Fig. 8A and B).

Next, we performed Mendelian Randomization analysis. Unfortunately, only NRCAM had a sufficient number of significant SNPs. MR Egger, Weighted Median, Inverse Variance Weighted (IVW), Simple Mode, and Weighted Mode all suggested that NRCAM was negatively correlated with AD risk (Fig. 8C and D). The results of the Weighted Median method were statistically significant ($P = 0.017$, OR = 0.999, 95% CI: [0.997–1.000]). Cochran's Q statistic indicated no heterogeneity among the IVs used in the analysis ($P > 0.05$). Although the results of IVW were not statistically significant ($P = 0.055$), these results suggest that NRCAM is a protective factor against AD, consistent with our findings using the ADNI cohort.

In the peripheral blood transcriptome, the expression levels of NRCAM and PYY were significantly downregulated in the AD patient group ($P < 0.01$), with no significant differences for the other genes (Fig. 8E). In the brain transcriptome, the expression levels of APOE, CHGA, and NRCAM were significantly downregulated in the AD patient group ($P < 0.0001$), with no significant differences for the other genes (Fig. 8F). Interestingly, although PYY expression did not differ significantly between the AD patient and control brain samples, the expression

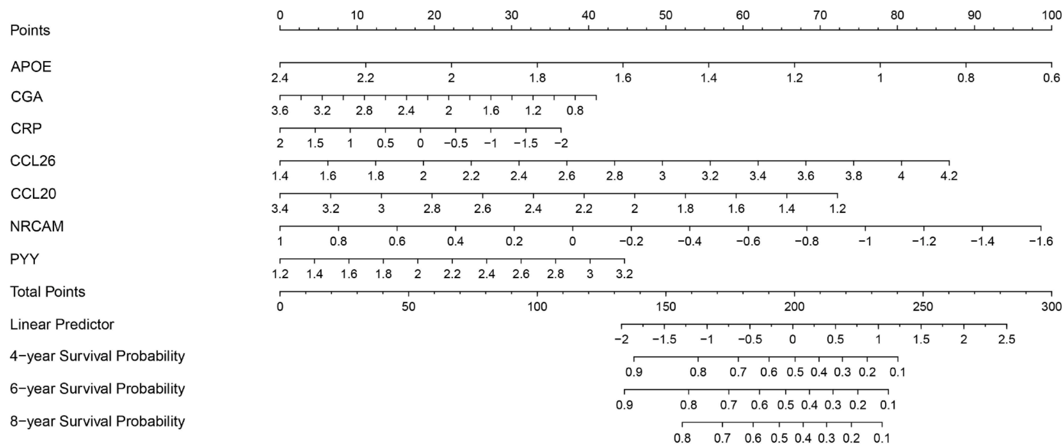

Fig. 2. Predictive nomogram was constructed using selected protein signatures.

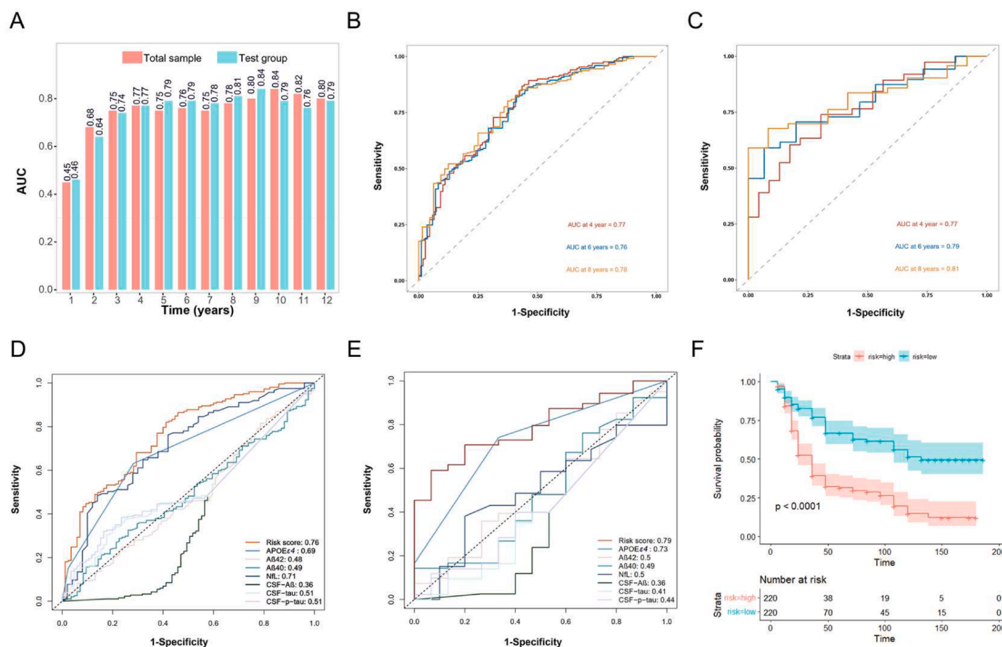

Fig. 3. The AUC of t-ROC curves of the predictive model remained stable over the 1st-12th year follow-up period in the total sample and test group (**A**). The t-ROC curves of the predictive model for AD at 4, 6, and 8 years in the total sample (**B**) and the test set (**C**). The model's AD risk prediction at 6 years (**D**) in the overall sample and the test set (**E**) had a higher AUC compared to CSF or other plasma biomarkers. For Kaplan-Meier analysis (**F**), the low-risk group showed a significantly lower AD incidence compared to the high-risk group ($P < 0.0001$).

of main receptor of PYY in the brain, NPY2R, was significantly downregulated in the AD patient group ($P < 0.001$). Combined with the peripheral blood analysis, the PYY-NPY2R signaling axis was overall suppressed in AD patients.

4. Discussion

This research introduced a new method utilizing plasma proteins to predict the risk of Alzheimer's disease incidence. We developed a concise predictive model for AD incidence by selecting seven specific plasma protein markers through Cox regression analysis, LASSO regression, and cross-validation. Evaluation through the t-ROC curve and Kaplan-Meier curve analysis demonstrated that our model exhibited strong predictive performance for AD incidence. Further analysis revealed that these proteins have the potential to be used as independent predictive biomarkers for AD.

In comparison to PET and CSF, blood testing is less invasive and more cost-effective, making it a more feasible option as a screening tool for large populations (Henriksen et al., 2014). Hence, the development of plasma protein biomarkers for AD holds promising potential. Previous research had attempted to develop early predictive models for AD onset utilizing plasma proteins. For example, using ApoB, calcitonin, and 12 other plasma proteins to predict the risk of MCI patients converting to AD within 4 years. (Araújo et al., 2022).Using levels of P-S396-tau, P-T181-tau, and Aβ1–42 in blood exosome extracts to predict the development of AD within 10 years. (Fiandaca et al., 2015) Using blood protein biomarkers to predict the 5-year risk of new-onset AD in patients with clinical subjective cognitive complaints (SCC) or MCI (Planche et al., 2023) .However, these researches primarily focused on binary classification at a fixed observation time rather than providing insights into AD incidence. In reality, it is more crucial to determine whether and when an individual will develop AD and quantify the associated risk indicators (Yang et al., 2022, Hou et al., 2023, Ge et al., 2023). This information not only aids in the dynamic assessment of the complex process of the development of AD but also provides more reliable support for interpreting indicators, interventions, and long-term

Table 3
Analysis of differences between the high-risk and low-risk groups.

Characteristics	High risk	Low risk	P value
N	220	220	
AD onset, n (%)	145(65.9%)	71(32.3%)	< 0.001
Age, mean (SD), years	74.1±7.2	75.4±7.0	0.047
Male gender, n (%)	153(69.5%)	123(55.9%)	0.003
Education years, mean (SD), years	15.7±3.0	15.6±3.0	0.692
APOE $\varepsilon 4$ carriers, n (%)	151(68.6%)	61(27.7%)	< 0.001
MMSE scores, mean (SD)	25.7±5.4	27.3±3.7	0.005
CDR-SB scores, mean (SD)	3.4±4.6	2.1±3.2	< 0.001
CSF-Aβ, mean (SD), pg/mL	403.8±504.2	650.9±661.4	< 0.001
CSF-tau, mean (SD), pg/mL	160.6±179.0	150.8±156.6	0.541
CSF-p-tau, mean (SD), pg/mL	16.0±18.5	14.2±15.8	0.290
Aβ42, mean (SD), pg/mL	35.5±12.7	36.6±12.3	0.371
Aβ40, mean (SD), pg/mL	148.3±59.7	150.8±49.3	0.637
NfL, mean (SD), pg/mL	41.9±28.0	39.9±29.8	0.583
CSF-APOE, mean (SD), ug/mL	22.6±0.9	22.4±0.8	0.092
CSF-CGA, mean (SD), ng/mL	15.0±1.2	15.6±1.3	0.001
CSF-CRP, mean (SD), ug/mL	34.1±0.8	34.0±0.8	0.402

monitoring. In our research, we constructed a Cox proportional hazards regression model based on plasma protein signature levels at baseline to quantified AD incidence. The validation results demonstrated that our risk predictive model remained relatively reliable and stable from the 3rd to the 12th year of observation (AUC $\geq$ 0.74). Based on our estimates, the stability of the model is likely attributed not only to its good fitting performance but also to the fact that LASSO regression and Cox regression account for time-dependent factors, capturing key protein signatures associated with long-term AD risk. These protein signatures, as risk-related factors for AD, exhibit relatively stable expression before the onset of the disease. This stability has further demonstrated that biological changes in plasma have long existed before the onset of AD, which generated significant interest in further investigating these proteins. In summary, using this strategy, interventions can be conveniently and precisely provided based on the estimated risk level.

Analyzing the relationship between plasma protein expression levels and the time of AD onset is crucial (Planche et al., 2023), as it provides insights into the independence of the selected protein signature. The use of LASSO regression and cross-validation methods helps address multicollinearity (Zhao et al., 2022), indicating that the protein expression levels chosen are independent of each other, which is confirmed by Pearson correlation analysis. The Kaplan-Meier curves of the protein signature reveal that each protein can serve as an independent predictive factor for AD risk, independent with AD CSF and traditional plasma biomarkers. This suggests that these protein signatures may offer potential for exploring the complex process of the development of AD from different perspectives and uncovering new potential mechanisms of AD onset through peripheral blood.

To further validate the relationship between these protein signatures and AD, we conducted follow-up verification using public databases. Colocalization analysis indicated a strong genetic correlation between AD and APOE. Mendelian randomization analysis suggested that NRCAM acts as a protective factor against AD. Peripheral blood transcriptome analysis revealed that the expression levels of NRCAM and PYY were significantly downregulated in the AD patient group. Brain transcriptome analysis showed that the expression levels of APOE, CGA, and NRCAM were significantly reduced in the AD patient group, and it was inferred that the PYY- NPY2R signaling axis in AD patients might be suppressed.

Among the identified plasma protein signatures, APOE is particularly noteworthy. The APOE gene comprises three alleles: $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$, with $\varepsilon 4$ being a risk factor for AD (Sun, Wang and Huang, 2023). Our results consistently show that plasma APOE is one of the most significant protective factors for AD risk and is negatively correlated with the APOE $\varepsilon 4$ genotype. In the central nervous system, APOE protein plays a crucial role in maintaining and repairing neurons (Koutsodendris, Nelson, Rao and Huang, 2022). CSF APOE $\varepsilon 4$ has been implicated in inducing AD through blood-brain barrier damage (Zhou et al., 2023). Previous research has indicated that low plasma APOE levels may be associated with various aspects of AD pathology, while high plasma APOE levels appear beneficial for neurodegenerative diseases (Giannisis et al., 2022, Liu et al., 2022), which aligns with our findings. Furthermore, we observed no correlation between plasma and CSF APOE levels at baseline, indicating that plasma and CSF APOE levels may contribute to increased AD risk through different mechanisms.

CGA, a member of the chromogranin protein family, is found in secretory vesicles in endocrine cells and neurons (Iyer et al., 2023). In the context of Alzheimer's disease, previous research has shown elevated levels of CGA in the temporal cortex, where it accumulates in amyloid plaques and dystrophic neurites (Quinn et al., 2023, Lechner et al., 2004, Venegas and Heneka, 2017). These plaques and neurites are associated with activated microglia, suggesting a potential role of CGA in the inflammatory response in AD. However, it is important to note that CGA production in the central nervous system is primarily local and may not directly correlate with peripheral blood levels (Popp et al., 2017). Interestingly, despite the lack of research investigating the relationship between blood CGA levels and AD, our research discovered that plasma CGA levels are protective against AD. This finding suggests that peripheral CGA may play a role in preventing AD. Furthermore, existing research has indicated that peripheral CGA and its derived peptides are involved in maintaining vascular homeostasis (Wei et al., 2021). For instance, Wang et al. demonstrated that a CGA-derived peptide, CGA47–66, can mitigate the disruption of occludin in the blood-brain barrier, improve cognitive impairments in septic mice, and protect against brain injury (Wang et al., 2022). Considering that blood-brain barrier disruption is considered a crucial feature of early AD, we speculate that the protective effect of plasma CGA against AD may be mediated through the preservation of the blood-brain barrier. Further investigation is warranted to unravel the underlying mechanisms connecting blood CGA levels and AD and to determine the potential therapeutic implications for targeting CGA in AD prevention.

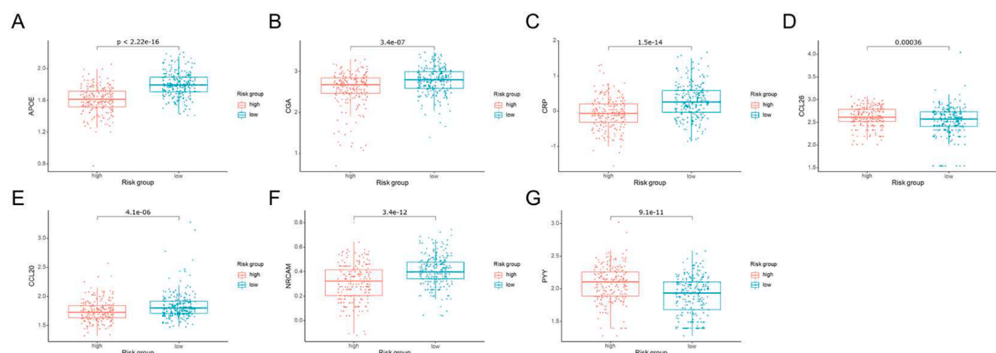

Fig. 4. The expression analysis of protein signatures in different risk groups of AD.

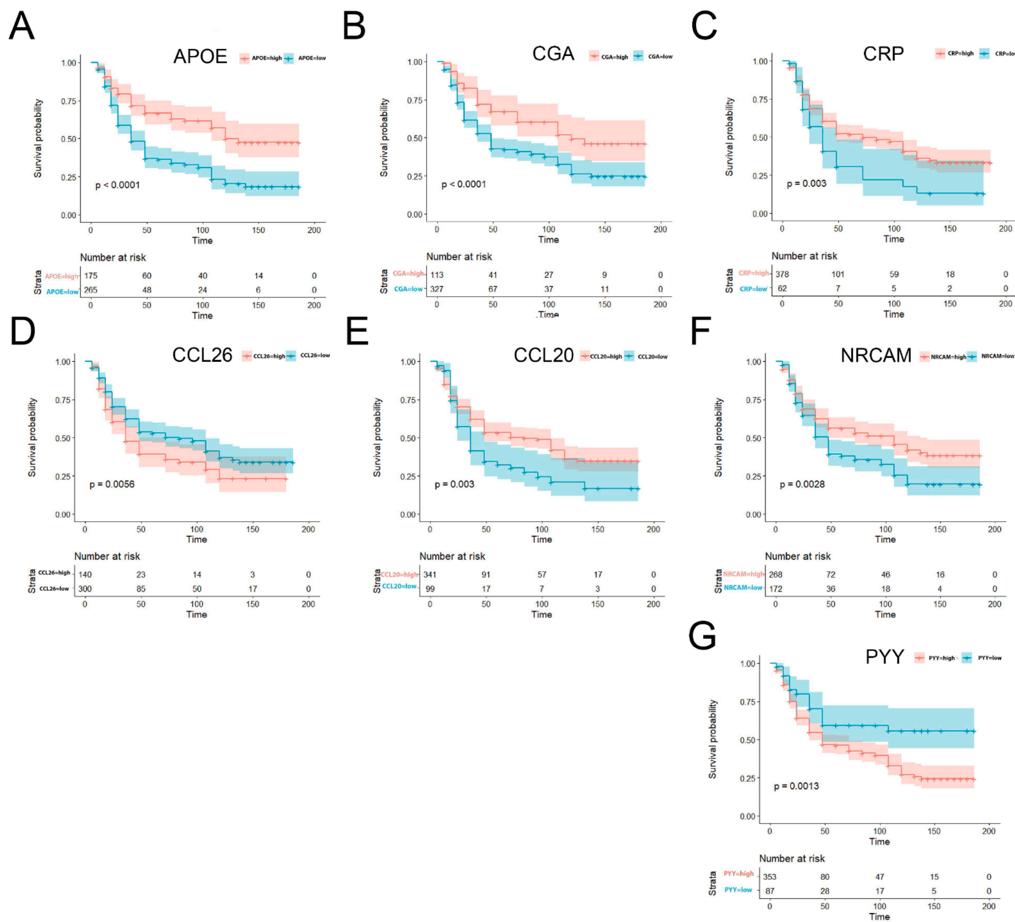

Fig. 5. Kaplan-Meier analysis of protein signatures with respect to AD onset. High expression of APOE (A), CGA (B), CRP (C), CCL20 (E), and NRCAM (F) is associated with a later AD onset, while high expression of CCL26 (D) and PYY (G) is associated with an earlier AD onset.

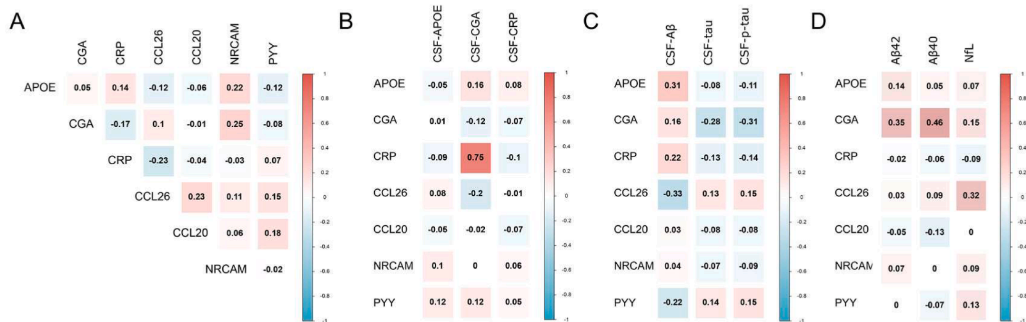

Fig. 6. Pearson correlation analysis of protein signature expression in plasma (A), the expression of protein signatures in plasma and APOE, CGA, and CRP in CSF (B), Aβ, tau, and p-tau in CSF (C) and Aβ42, Aβ40, NfL in plasma (D). The redder the color, the greater the correlation coefficient.

CRP is a widely used inflammatory marker that is generally associated with an increased risk of various diseases (Cardoso et al., 2015, Memar, Alizadeh, Varshochi and Kafil, 2019). However, contrary to expectations, some research has shown that CRP levels are decreased in the early and middle stages of AD compared to healthy controls (Gong et al., 2016). Furthermore, lower CRP levels have been associated with faster cognitive and functional decline in individuals with AD. Interestingly, CRP levels in the blood have been found to be negatively correlated with APOE *ε4* genotype, a genetic risk factor for AD. These findings suggest that CRP may play a long-term role in the progression of AD, especially in individuals with a genetic predisposition. However, the exact mechanisms underlying the association between CRP and AD remain unclear (Zhang et al., 2023). Further research is needed to explore the role of CRP in the risk and progression of AD.

CCL20 (macrophage inflammatory protein 3α, MIP3α) is involved in leukocyte activation, chemotaxis, and migration, playing a vital role in inflammation and immune responses. Previous studies have shown that CCL20 plays a crucial role in neurodegenerative changes following trauma in rodents (Das et al., 2019). Knockout of CCL20 results in reduced activation of microglia and decreased neuroinflammation (Hu, Yang, Li and Lu, 2016), suggesting that CCL20 may mediate neurodegenerative and inflammatory effects. Additionally, CCL20 can recruit Treg cells and regulate their infiltration into the brain (Rutihinda et al., 2023), further supporting its role in mediating central nervous system inflammation. In our research, we found that CCL20 acts as a protective factor against AD (Meitei, Jadhav and Lal, 2021). It has been reported

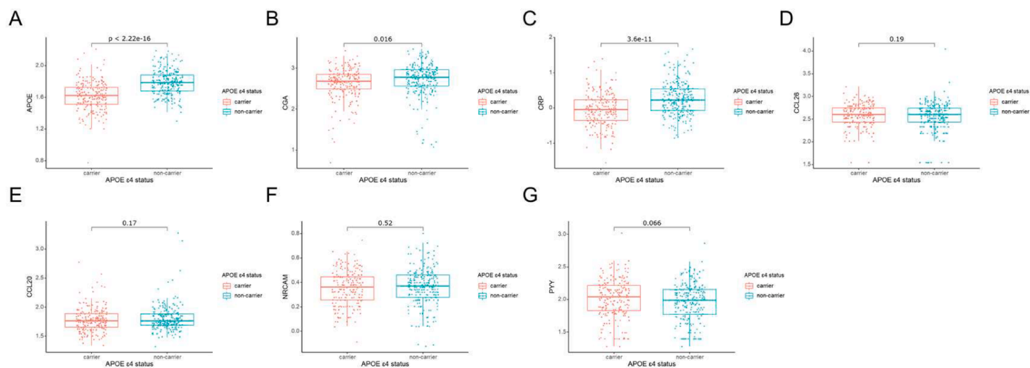

Fig. 7. Expression analysis showed significantly lower levels of APOE (A), CGA (B), and CRP (C) ($P<0.05$) in APOE$\varepsilon 4$-positive individuals. The expression levels of other proteins (D-G) did not show significant statistical significance.

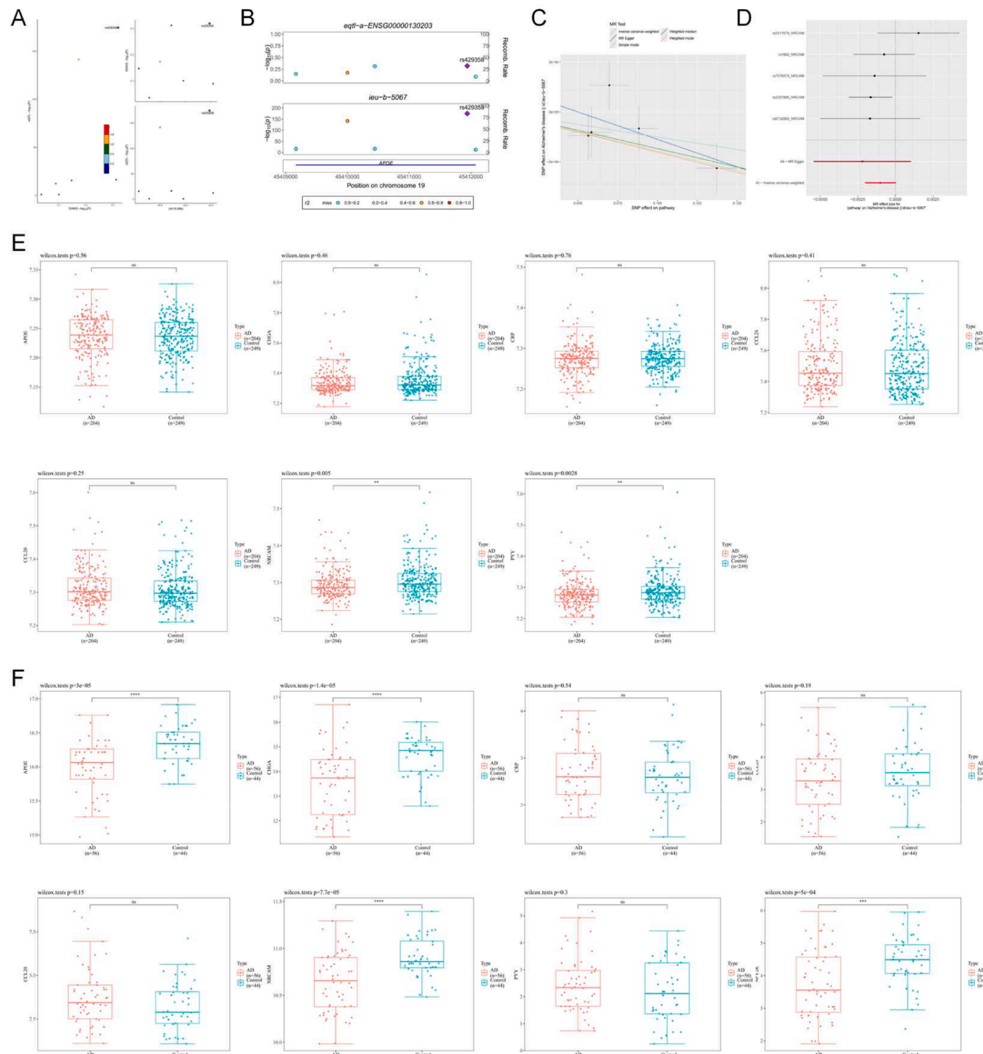

Fig. 8. Colocalization analysis using the locuscompare R package for visulization (A). The labeled SNP is the lead SNP (in this case for both studies), and other SNPs are colored according to their Linkage Disequilibrium (LD) with the lead SNP. Colocalization analysis using the gassocplo R package for visulization (B). The purple diamond represents the minimum P value SNP corresponding to eQTL and GWAS. Scatter plot shows the relationship between AD and NRCAM (C). Each dot represents a SNP. The horizontal coordinate indicates the effect size of the SNP, and the vertical coordinate indicates the effect size of the exposure factor. The colored lines represent the MR Fitting results. Forest map shows overall effects between NRCAM and AD (D). Boxplots show the expression distribution of gene signatures in blood and brain (E-F).

that plasma from AD patients can inhibit the generation of CCL20 in vitro (Shim, Kim and Jeon, 2017). Additionally, the expression of CCL20 has been found to decrease in the plasma of AD mouse models (Sun et al., 2020). These findings imply that CCL20 may have a potential therapeutic role in AD by modulating inflammation and immune responses.

NRCAM is a member of the immunoglobulin superfamily and is involved in various neuronal functions, including cell adhesion, brain wiring, and myelination (Bai and Chen, 2022, Sakurai, 2012). NRCAM is expressed in neurons and glial cells in both the central and peripheral nervous systems, where it regulates signaling pathways in neurodevelopment through interactions with various guidance and other molecules (Sakurai, 2012). Its role has been identified in the pathogenesis of various conditions, including autism, gliomas, addiction, and anxiety (Baran et al., 2022). Research conducted on Nrcam knockout mice has shown that the absence of Nrcam leads to impaired social behavior and cognitive function (Ishiguro et al., 2014). NRCAM has the potential to be used as a biomarker in CSF for AD (Hu et al., 2010). Furthermore, NRCAM may serve as an indicator of the selective activation of the metalloproteinase ADAM10, involved in the cleavage of amyloid precursor protein (APP), suggesting it may play a protective role in AD and could be a potential therapeutic target for AD (Brummer et al., 2019) . Our model results also indicate that NRCAM is a potential protective factor in AD, validated through Mendelian randomization analysis. Additionally, our peripheral blood transcriptome confirmed that NRCAM expression is significantly downregulated in the AD patient group. These findings further strengthen the evidence of NRCAM's protective role in AD onset.

CCL26 (Eotaxin 3) belongs to the CC chemokine family has been found to be dysregulated in the CSF of AD patients and differentiate AD from other neurodegenerative diseases, highlighting its potential as a specific biomarker for AD. (Huber, Giles, Segal and Irani, 2018). Elevated levels of CCL26 have also been observed in the CSF of individuals in the prodromal stage of AD (Goudey, Fung, Schieber and Faux, 2019), and these levels are correlated with age and CSF tau protein levels (Westin et al., 2012). In individuals with Down syndrome, CCL20 levels increase before the development of significant AD pathology (Flores-Aguilar et al., 2020). Additionally, CCL26 is elevated in the temporal cortex of asymptomatic individuals with Aβ pathology and is associated with immune responses to pathological tau (Flores-Aguilar et al., 2021). This suggests that CCL26 may be involved in the early stages of AD development and progression. Our study suggests that CCL26, consistent across brain, CSF, and blood samples before AD conversion, may serve as a risk factor and biomarker for AD onset.

PYY is a member of the pancreatic polypeptide family that can be secreted by the gastrointestinal tract into the bloodstream (Chen et al., 2023). It is capable of crossing the blood-brain barrier and has receptors in brain regions associated with AD (Domingues et al., 2018). Previous research has shown that increased levels of PYY in healthy older individuals are associated with decreased brain volume, suggesting negative effects on the brain (Morris et al., 2020). However, this phenomenon was not observed in AD patients (Ahmed et al., 2015), indicating that PYY may be involved in pre-AD pathological changes and may not play a significant role after severe neurodegeneration has occurred. Identifying and exploring protein signatures with such characteristics, which are often overlooked in traditional research, can be achieved through risk predictive models. This approach provides new directions for the search for AD biomarkers.

From both mechanistic and clinical perspectives, this study has identified promising new plasma protein biomarkers and developed a promising AD risk prediction model. Plasma samples can be rapidly collected in clinical settings or community clinics and quantified for protein signatures using convenient detection technologies such as enzyme-linked immunosorbent assay (ELISA). Based on formulas or nomograms, physicians can calculate the risk of AD occurrence in the coming years, guiding individuals to make preventive health decisions, such as encouraging high-risk populations to engage in social activities, cognitive training, and physical exercise to prevent AD. However, there are still some limitations to overcome before our model can be applied in clinical practice. It is necessary to expand sample sizes, extend follow-up periods, continuously optimize protein signature selection and prediction models, and further investigate the biological mechanisms linking these plasma proteins with AD development.

5. Conclusion

Our research identifies plasma proteins independently associated with the risk of AD and developed a risk prediction model based on these proteins. Furthermore, the plasma protein signature we have identified may be linked to the risk of AD through different pathways, suggesting their potential as new biomarkers for AD onset. Further research is needed to delve deeper into the mechanisms underlying the development of AD and to explore how these plasma proteins can provide valuable information for the prevention and treatment of AD from a blood-based perspective. However, our model was constructed using data solely from the ADNI database, which has certain limitations.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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CRediT authorship contribution statement

Tianchi Zhuang: Writing – review & editing, Writing – original draft, Methodology. **Yingqi Yang:** Writing – original draft. **Haili Ren:** Writing – review & editing. **Haoxiang Zhang:** Visualization. **Chang Gao:** Visualization. **Shen Chen:** Formal analysis. **Jiemiao Shen:** Writing – review & editing, Visualization. **Minghui Ji:** Writing – review & editing, Supervision. **Yan Cui:** Supervision.

Declaration of competing interest

The authors declare that they have no competing interests.

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organization is the Northern California Institute for Research and Education, and the study is coordinated by the Alzheimer's Disease Cooperative Study at the University of California, San Diego. ADNI data are disseminated by the Laboratory for Neuro Imaging at the University of California, Los Angeles.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.archger.2024.105650.

Data availability

The datasets generated and/or analyzed during the current study are available in the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu).

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