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Mitochondria-Related Candidate Genes and Diagnostic Model to Predict Late-Onset Alzheimer's Disease and Mild Cognitive Impairment

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Abstract.
Background: Late-onset Alzheimer's disease (LOAD) is the most common type of dementia, but its pathogenesis remains unclear, and there is a lack of simple and convenient early diagnostic markers to predict the occurrence.
Objective: Our study aimed to identify diagnostic candidate genes to predict LOAD by machine learning methods.
Methods: Three publicly available datasets from the Gene Expression Omnibus (GEO) database containing peripheral blood gene expression data for LOAD, mild cognitive impairment (MCI), and controls (CN) were downloaded. Differential expression analysis, the least absolute shrinkage and selection operator (LASSO), and support vector machine recursive feature elimination (SVM-RFE) were used to identify LOAD diagnostic candidate genes. These candidate genes were then validated in the validation group and clinical samples, and a LOAD prediction model was established.
Results: LASSO and SVM-RFE analyses identified 3 mitochondria-related genes (MRGs) as candidate genes, including *NDUFA1, NDUFS5,* and *NDUFB3.* In the verification of 3 MRGs, the AUC values showed that *NDUFA1*, *NDUFS5* had better predictability. We also verified the candidate MRGs in MCI groups, the AUC values showed good performance. We then used NDUFA1, NDUFS5 and age to build a LOAD diagnostic model and AUC was 0.723. Results of qRT-PCR experiments with clinical blood samples showed that the three candidate genes were expressed significantly lower in the LOAD and MCI groups when compared to CN.

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Conclusion: Two mitochondrial-related candidate genes, NDUFA1 and NDUFS5, were identified as diagnostic markers for LOAD and MCI. Combining these two candidate genes with age, a LOAD diagnostic prediction model was successfully constructed.

Keywords: Alzheimer's disease, biomarker, late-onset Alzheimer's disease, immune cells, machine learning, mild cognitive impairment, mitochondria related genes

INTRODUCTION

Alzheimer's disease (AD), the most common form of dementia, is characterized by memory loss and cognitive impairment. Most cases occur after the age of 65, constituting late-onset AD (LOAD), while less than 5% of all cases occur earlier than age 65, which is termed early-onset AD (EOAD) [1]. The current leading hypotheses, the amyloid and tau propagation hypotheses, state that pathological tau and amyloid-β (Aβ) deposits are involved in triggering cascade reactions that occur in the cerebral cortex of patients with AD [2–4]. However, the underlying mechanism remains unclear, and many failures in clinical trials based on Aβ plaques or tau tangles have led to doubt on the hypotheses [5]. In addition to these two mainstream hypotheses, other hypotheses such as the cholinergic [6], mitochondrial cascade and related hypotheses [7–10], synaptic degeneration [11], and inflammatory [12, 13] hypotheses are also important possible explanations for the mechanisms underlying AD.

The onset of AD is insidious [2, 14], and many pathological changes occur before reaching clinical diagnostic criteria [15, 16]. Early detection and treatment of the disease are of great significance for delaying the development of dementia and improving its prognosis. Mild cognitive impairment (MCI) is an important component of predementia. People with MCI have subtle symptoms, such as problems with memory, language, and thinking, and these problems may not interfere with their ability to carry out everyday activities [14]. The cumulative dementia incidence in individuals with MCI older than 65 years who are monitored for two years is 14.9% [17]. Early screening and intervention for MCI is of great significance in the progression of dementia.

The current biological staging model for AD is based on the Aβ-tau-neurodegeneration (ATN) classification system, which assesses three biomarkers: Aβ, tau pathology, and neurodegeneration or neuronal injury [18]. Blood-based markers have emerged as a promising tool for the diagnosis of AD and for improving the design of clinical trials. The $A\beta_{42}/A\beta_{40}$ ratio and phosphorylated tau have shown potential as blood-based AD biomarkers [19]. However, detecting plasma Aβ and tau presents several challenges, including the expense and slow detection methods such as mass spectrometry and immunoassay, and potential inaccuracies in measurement due to pre-analytical processing and analytical performance [20].

Genetic and genomic analyses are becoming increasingly important in biomedical research because they can reveal the potential modes of action and mechanisms of diseases at the molecular level [21]. At present, there have been some bioinformatics studies on differential gene expression in peripheral blood cells of patients [22–25], including ferroptosis [23] and immune factors [24]. However, most studies have not specifically analyzed gene expression in LOAD, the main subtype of AD.

To explore and identify potential biomarkers of LOAD, public datasets GSE63060, GSE63061, and GSE140829 from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database were used. Using differential expression analysis, least absolute shrinkage selection operator (LASSO), and support vector machine recursive feature elimination (SVM-RFE) analysis, two candidate mitochondria-related genes (MRGs) were identified and used to establish a LOAD prediction model. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were used to further investigate biological processes and pathways. Then, the cell-type identification by estimating relative subsets of RNA transcripts (CIBERSORT) algorithm was applied to calculate the immune infiltration of LOAD samples. The workflow of this study is shown in Fig. 1.

MATERIALS AND METHODS

Data acquisition

The peripheral blood gene expression data used in this study were obtained from the NCBI GEO

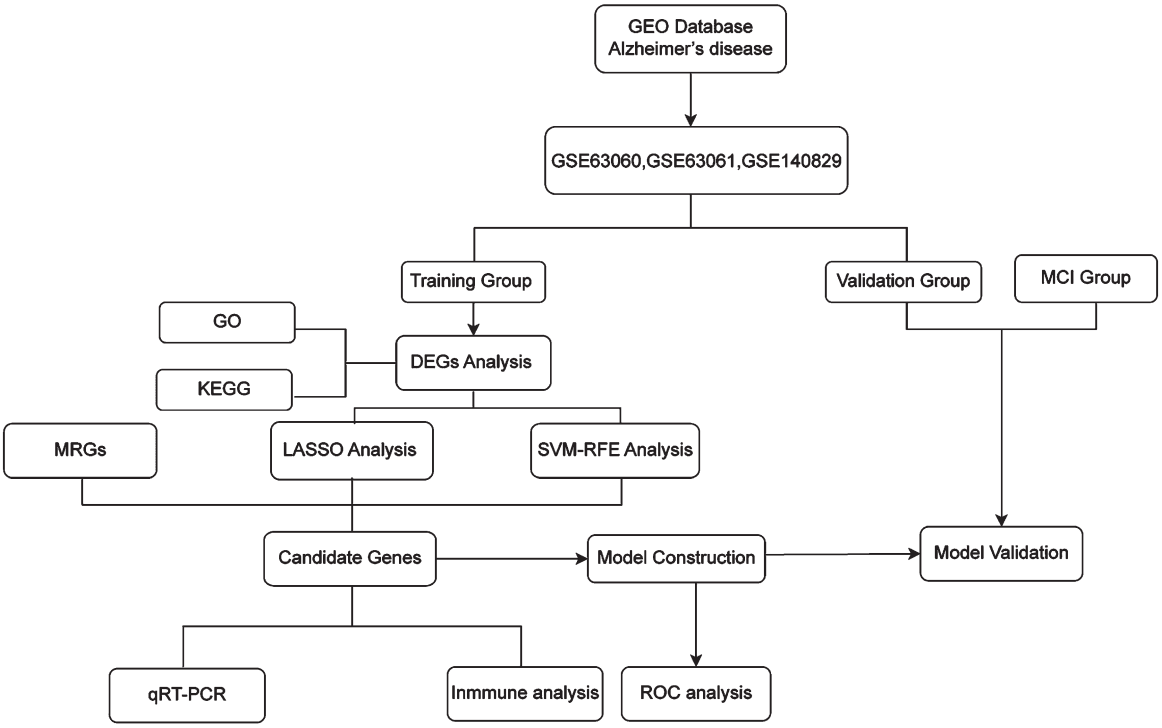

Fig. 1. The workflow of the analysis, including data extraction, processing, and analysis.

database [26]. As LOAD typically occurs after the age of 65, samples from individuals ≤65 years of age were excluded from the analysis. Ultimately, we used three data series for analysis: GSE63060 annotated by GPL6947, which included 134 LOAD samples, 80 MCI samples, and 94 cognitively normal (CN) samples; GSE63061 annotated by GPL10558, which included 133 AD samples, 104 MCI samples, and 131 CN samples; and GSE140829 annotated by GPL15988, which included 168 AD samples, 116 MCI samples, and 229 CN samples. All samples were obtained from individuals over 65 years of age. To perform our analysis, we randomly split the LOAD and CN samples in each data series into 3 : 1 as training and validation groups, respectively. We assigned all 300 MCI samples to the MCI validation group.

The studies involving human participants were reviewed and approved by the Ethics Committee of the Ruijin Hospital affiliated to the Shanghai Jiao Tong University School of Medicine (2018-No.204).

Differential expression analysis

Differential expression analysis of LOAD and CN samples was performed using the "limma" R package [27]. Differentially expressed genes (DEGs) (p_adjust < 0.01) were obtained, volcano plots of the DEGs were created using the "pheatmap" [28] and "ggplot2" R packages.

Bio-functional analysis

To investigate which biological pathways the DEGs in LOAD are involved, we conducted functional enrichment analyses. Using R package "clusterProfile" [29] and "enrichplot" [30] R packages, GO analysis which focuses on three levels including cell component (CC), biological process (BP), and molecular function (MF), and KEGG analysis which is mainly used for pathway enrichment analysis were performed on the DEGs.

LASSO and SVM-RFE analysis

To further identify the diagnostic candidate genes for LOAD from these DEGs, we performed the following two machine learning methods for further screening. LASSO regression analysis was fitted using the "glmnet" package [31], set the "family" parameter as "binomial" and the "alpha" as 1, the cross-validation parameter "nfolds" was adjusted to 10. SVM-FRE is a sequential backward selection

algorithm based on the maximum interval principle of SVM. It trains the sample through the model, then sorts the score of each feature, removes the feature with the minimum score, and then trains the model again with the remaining features until selects the required number of features. The SVM-RFE classifiers from R packages "e1071" [32], "kernlab" [33], and "caret" were adopted for the classification analysis of the selected candidate genes in the diagnosis of AD.

Identification of MRG candidates

In this study, MRGs refer to the genes that encode proteins located in any part of the mitochondria including the mitochondrial membrane, stroma, cristae, and mitochondria-associated endoplasmic reticulum. The MitoCarta 3.0 database included 1,136 human mitochondria-located genes [34, 35], and the MRGs list was downloaded for subsequent analysis (Supplementary Table 1).

Immune infiltration and immune-related factors

To evaluate the immune infiltration of LOAD peripheral blood, we applied the CIBERSORT algorithm. CIBERSORT [36] performed deconvolution analysis based on the principle of linear support vector regression, and there were 22 types of immune cells provided, including plasma cell, B cell, T cell, and myeloid cell subpopulations. We used this algorithm to analyze the gene expression data of the training set and calculate the relative proportions of each type of immune cells in each sample. Spearman correlation analysis was used to analyze the correlation between candidate genes and immune cells.

Model construction and evaluation

To assess the ability of candidate genes to distinguish disease states, the receiver operating characteristic curve (ROC) was plotted by "pROC" [37]. ROC could reflect the trend of sensitivity (FPR) and accuracy (TPR) of the model when different thresholds were selected, and the value of the area under the curve (AUC) can be used as an evaluation index. We tested the AUC of candidate genes on the training set data, and subsequently tested them on the MCI and LOAD validation set data. In order to improve the accuracy of disease diagnosis, we combined the two candidate genes with the highest accuracy and age to construct a multi-factor disease prediction model, which was assessed in 3 ways. In addition to the ROC method, calibration curve was plotted to present how close the actual incidence is to the predicted incidence calculated by LOAD prediction nomogram. Considering the impact of false positives and false negatives on patients, the concepts of threshold probability and net benefit are introduced in decision curve analysis (DCA), which was used to assess the benefit of patients using our predictive model in the clinic.

qRT-PCR validation of the candidate genes

Peripheral blood samples of 8 participants who were CN, 8 patients with LOAD, and 10 patients with MCI were acquired for qRT-PCR to verify the expression of candidate genes. Diagnosis was based on NIA-AA Research Framework [14]. Participants were over 65 years old and underwent neuropsychological assessments, including Mini-Mental State Examination (MMSE), Montreal Cognitive Assessment (MoCA), and Clinical Dementia Rating Scale (CDR). Brain magnetic resonance imaging and PET-CT in LOAD and MCI were performed to help diagnose. This study was approved by the Ethics Committee of the Ruijin Hospital affiliated to the Shanghai Jiao Tong University School of Medicine (2018-No.204). The RNAprep Pure Hi-Blood Kit (DP443, TIANGEN) was used to extract total RNA, RNA quality was determined by TGem Plus full-wavelength spectrophotometer (OSE-260-02, TIANGEN), A260/280, A260/230 absorbance ratios of purified RNA between 2.0–2.2, 1.8–2.2 respectively for subsequent experiments. RNA was then reverse-transcribed to cDNA and qRT-PCR was performed with the 2×Hieff® PCR Master Mix (10102ES08; Yeasen). GAPDH was used as an internal reference and the primers used are listed in Table 3. Relative mRNA expression was calculated using the $\Delta\Delta$Ct method.

Statistical analyses

The chi-square test was adopted for categorical data (expressed as a percentage), and measurement data were analyzed by *t*-test (represented by a mean±SEM). A logistic regression algorithm and SVM-RFE were used to construct the prediction model. All statistical analyses were performed using R language software (version 4.2.1) and GraphPad Prism 9. Statistical significance was defined as $p < 0.05$.

RESULTS

The DEGs in LOAD were related to mitochondria

To identify DEGs related to LOAD, we downloaded the GSE63060, GSE63061, and GSE140829 datasets from the NCBI GEO public database, selected samples > 65 years old to match the age of onset of LOAD, and then randomly split each data series into 3 : 1 as training and validation groups. In total, there were 353 people who were CN and 322 patients with LOAD in the training group, and 101 people who were CN and 113 patients with LOAD in the validation group. To explore the biomarkers of LOAD, we first obtained DEGs from training group (p_adjust < 0.01), 78 DEGs were obtained (Supplementary Table 2), and a volcano plot of these DEGs is shown in Fig. 2A. Most of the genes with altered expression were downregulated (blue dots), and interestingly, 40% of the TOP 10 downregulated genes were related to mitochondria (green dots), which suggested that mitochondria-related genes (MRGs) were associated with LOAD.

Pathway enrichment of DEGs was associated with mitochondrial function

To determine the potential biological roles of the selected DEGs, we performed enrichment analysis. Figure 2B shows the top 15 KEGG pathways (ribosome-related pathways were excluded due to low specificity), in which oxidative phosphorylation pathways changed significantly (q-value<0.0025). The selected DEGs are also involved in disease such as AD, Parkinson's disease, prion diseases, and multiple neurodegenerative diseases. GO analysis showed that the top 10 pathways changed significantly, and after excluding the ribosome-related pathways, the remaining altered pathways all involved the mitochondria (Fig. 2 C). Target genes were associated with the aerobic electron transport chain (ETC), adenosine triphosphate (ATP) synthesis coupled electron transport in BPs, respiratory chain complex, mitochondrial respirasome, respirasome, and inner mitochondrial membrane protein complex in CCs. In addition, DEGs were involved in MFs such as electron transfer activity and nicotinamide adenine dinucleotide (NADH) dehydrogenase (ubiquinone) activity. Interestingly, the two pathway enrichment analyses both pointed to mitochondrial function changes in LOAD, which indicated that mitochondrial dysfunction played an important role in molecular biological processes of LOAD.

Two MRGs were identified as candidate genes for LOAD and MCI

To screen for the most significant genes that can be used as candidate genes for the diagnosis of LOAD in the selected DEGs, machine learning methods, including feature screening through LASSO regression and SVM-RFE were performed. The results of the LASSO analysis are shown in Fig. 3, which highlighted that the model had minimal cross-validation error when $\lambda = 21$, and 21 genes were identified as signature genes in LOAD by LASSO analysis (Fig. 3A, B). Simultaneously, we used the SVM-RFE algorithm to evaluate the characteristic genes, which showed that the model incorporating 31 genes had the best accuracy (Fig. 3 C). Thus, SVM-RFE yielded 31 candidate genes. In addition, 40% of the TOP 10 downregulated genes were related to mitochondria, and two pathway enrichment analyses were involved in mitochondrial function, indicating that there were significant changes in MRGs in LOAD. Based on previous DEGs and enrichment analyses, we decided to focus on MRGs. To define the MRGs from our previous results, 1,136 mitochondria-located genes were downloaded from MitoCarta3.0. We then selected common genes from the LASSO analysis, SVM-RFE analysis, and MRGs. Finally, the common three MRGs, including *NDUFA1* (NADH: ubiquinone oxidoreductase subunit A1), *NDUFS5* (NADH dehydrogenase (ubiquinone) Fe-S protein 5), and *NDUFB3*(NADH: ubiquinone oxidoreductase subunit B3) were regarded as candidate genes for the ongoing study (Fig. 3D). The results showed that the LOAD predictive accuracies (AUC values) of the three candidate genes were 0.703 (*NDUFA1*, Fig. 4A), 0.701 (*NDUFS5*, Fig. 4B), and 0.594 (*NDUFB3*, Fig. 4 C) in the training group. *NDUFA1* and *NDUFS5* had better predictability, but *NDUFB3* was not effective. Next, we used the data from the validation group to verify the AUC of the two better-performing candidate genes, the expression of both genes was reduced in LOAD (Fig. 5A, B), and the AUC values of *NDUFA1* and *NDUFS5* were 0.687 and 0.682 (Fig. 5 C), respectively.

We were curious whether these candidate genes were altered in the MCI stage, which is the pre-AD stage. Therefore, we collected samples from all patients with MCI over 65 years old in the GSE63060, GSE63061, and GSE140829 datasets to test our

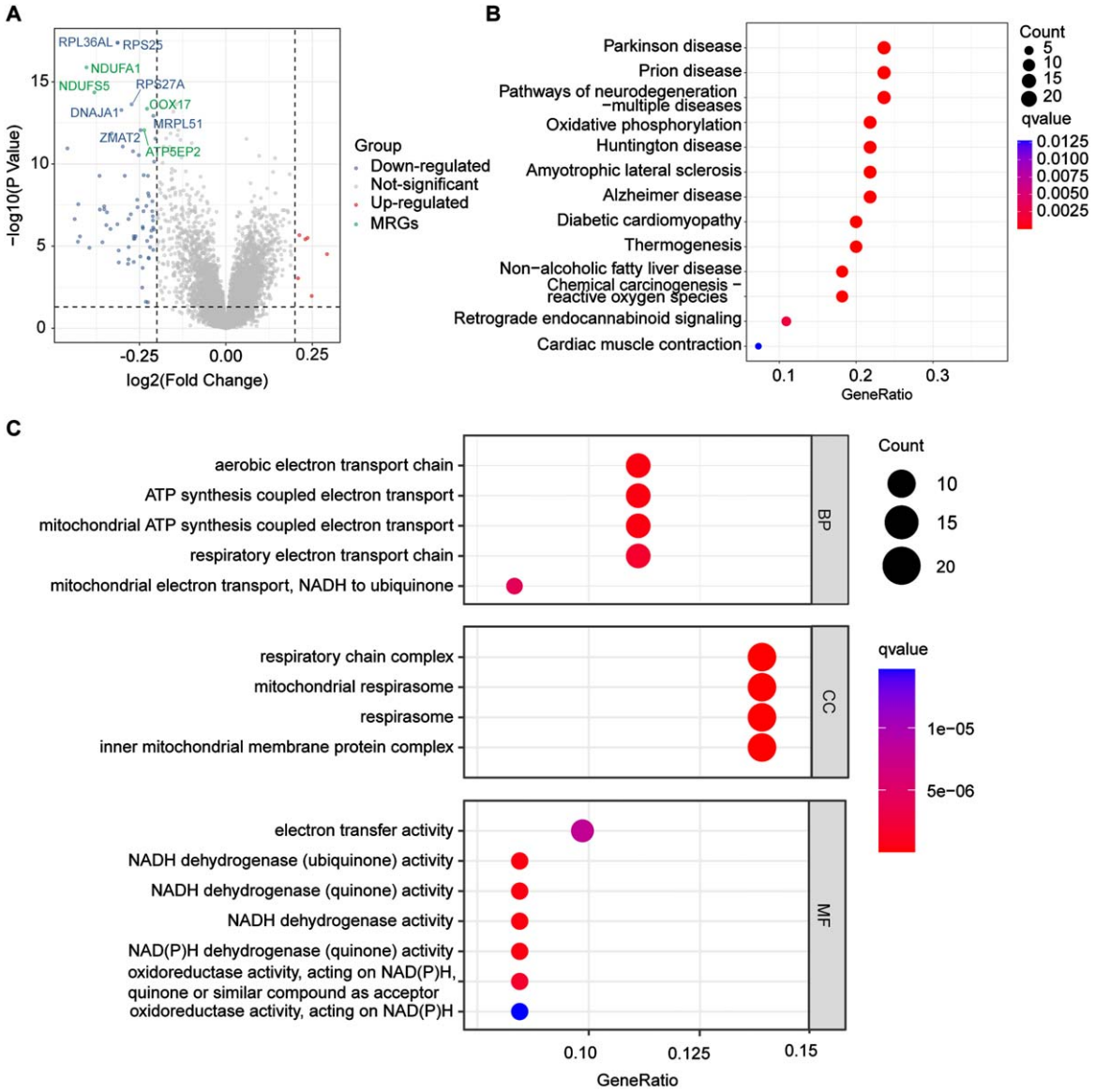

Fig. 2. Differentially expressed analysis. A) The volcano shows the top 10 genes significantly changed in LOAD groups, red dots and blue dots represent upregulated and downregulated genes in the LOAD group respectively, while green dots represent downregulated mitochondria-related genes in the LOAD group. LOAD, late-onset Alzheimer's disease. B) TOP 15 enriched KEGG pathways among LOAD DEGs. KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes. C) TOP 10 enriched GO pathways among LOAD DEGs. Ribosome-related pathways were removed due to low disease specificity. GO, Gene Ontology; BP, biological process, CC, cellular component; MF, molecular function.

hypothesis. In the analysis, 300 MCI samples were included. Similar to the results of LOAD, both genes were downregulated in MCI (Fig. 5A, B), and the AUC values of *NDUFA1* and *NDUFS5* were 0.668 and 0.652 (Fig. 5D), respectively. The number of participants and the AUC values in each group were summarized in Tables 1 and 2. The above results indicated that the two candidate MRGs had high accuracies as single factors to predict both LOAD and MCI.

Immune infiltration and immune-related factors changed in LOAD

Studies have shown that the pathogenesis of AD may be related to the infiltration, interaction, and

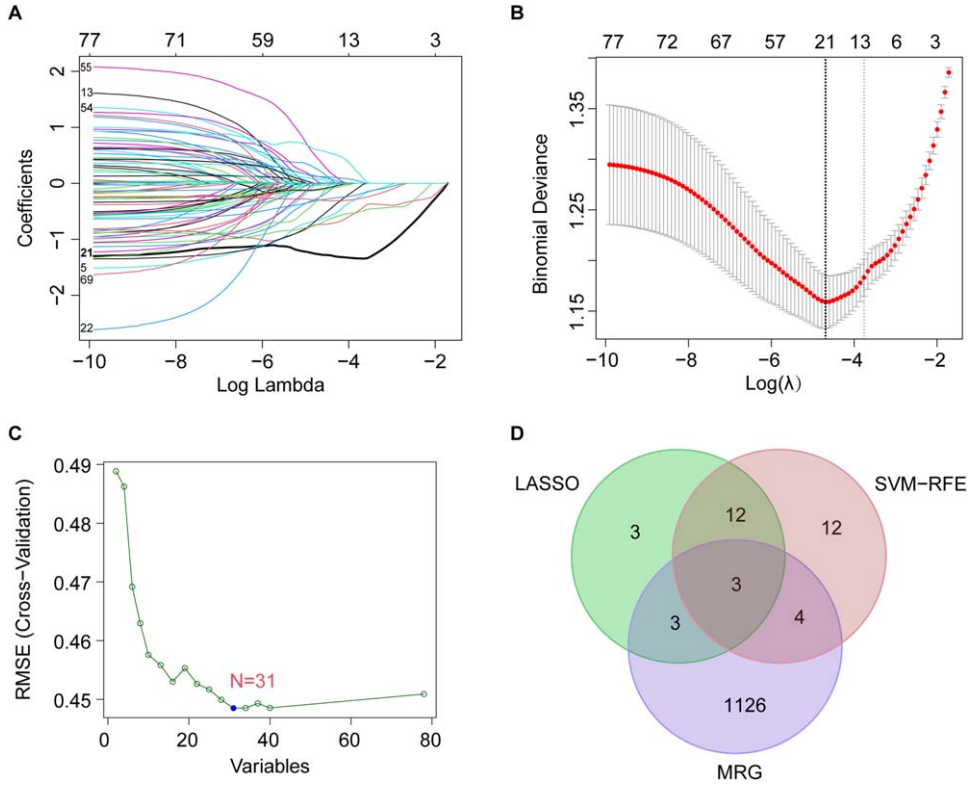

Fig. 3. Selection of diagnostic biomarkers and identification of candidate genes. A, B) The 21 genes that met the diagnostic criteria were determined by LASSO analysis. A) The horizontal axis represents the log value of the gene lambda, and the vertical axis represents the independent gene's coefficient. LASSO, least absolute shrinkage and selection operator. B) CIs with different values of lambda. C) 31 characteristic genes were identified by SVM-RFE algorithm. The horizontal axis represents the number of genes included, and the vertical axis represents the error of cross validation. SVM-RFE, Support Vector Machine Recursive Feature Elimination. D) Venn diagram of MRGs extracted from LASSO and SVM-RFE methods. MRG, mitochondria-related gene.

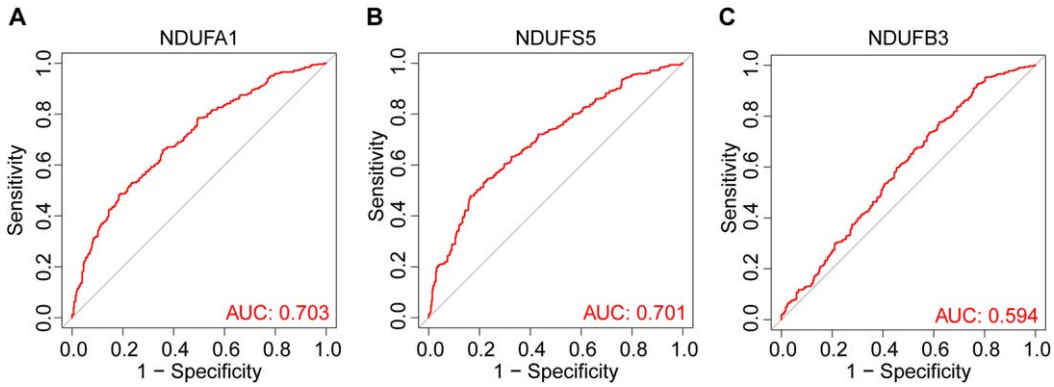

Fig. 4. ROC curves and corresponding AUC values for the training groups. The ROC curves of NDUFA1 (A), NDUFS5 (B), and NDUFB3 (C), AUC values were 0.703, 0.701, and 0.594 respectively.

dysfunction of immune cells [38, 39]. Studying the characteristics of immune cell infiltration in LOAD and the relationship between the candidate genes and immune cells will help to increase our understanding of the importance of immunity in LOAD and identify potential diagnostic and therapeutic targets.

In this study, the CIBERSORT algorithm was used to analyze 22 immune cell components in 322 LOAD

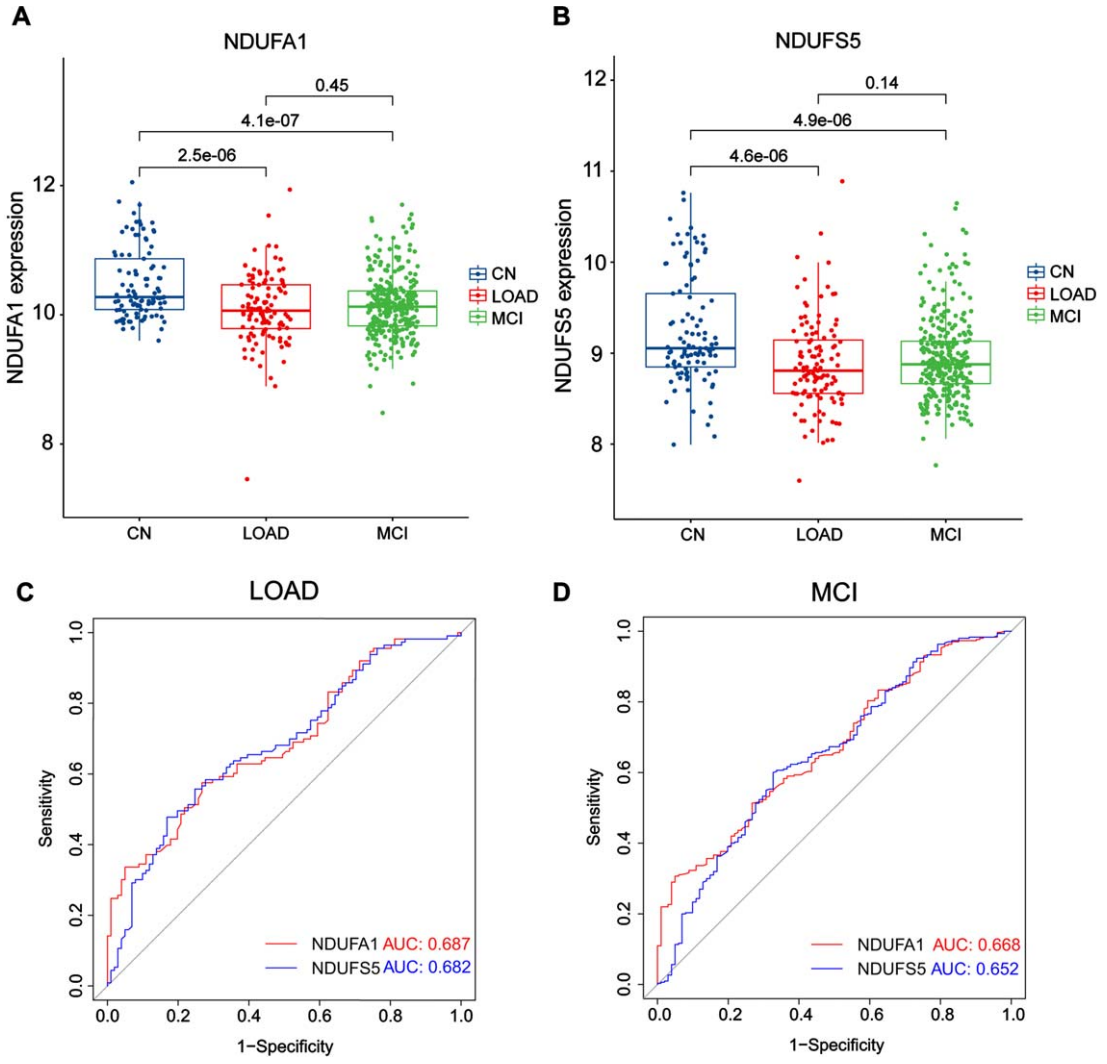

Fig. 5. Expression and corresponding AUC value of candidate genes in the CN, LOAD, and MCI groups in validation groups. The expression of NDUFA1 (A) and NDUFS5 (B) was significantly lower in LOAD and MCI. The ROC curves showed that the AUC values of NDUFA1 and NDUFS5 were 0.687 and 0.682 in the LOAD validation groups (C), and the AUC values of NDUFA1 and NDUFS5 were 0.668 and 0.652 in the MCI validation groups (D).

Table 1
The number of participants in each group

	CN	LOAD	MCI
Training Group	353	322	
Validation Group	101	113	300

Table 2
The AUC values in each group

	Training	Validation on LOAD	Validation on MCI
NDUFA1	0.703	0.687	0.668
NDUFS5	0.701	0.682	0.652
NDUFB3	0.594		

samples and 353 CN samples, the results are shown in the histogram (Fig. 6A). Immune cells with significant differences between groups were presented in a violin plot (Fig. 6B), which showed that the LOAD group had significantly higher proportions of regulatory T cells (Tregs) ($p = 0.010$) and gamma delta T cells ($p < 0.001$), and lower proportions of naüve B cells ($p < 0.001$) and resting CD4 memory T cells ($p < 0.001$). The proportion of immune cells in peripheral blood was altered in LOAD.

To further explore the relationship between the candidate MRGs and immune cells, we performed

Table 3
Primer information

Primers		Sequence
NDUFA1	F	ATGTGGTTCGAGATTCTCCCC
	R	CCTGTGGATGTACGCAGTAGC
NDUFS5	F	TGCACATGGAATCGGTTATACTC
	R	CCGAAGCAAACACTCTACGAAAT
NDUFB3	F	TGCTGTCAGGCAGAAGAACAG
	R	CTTAGCCCTTTTGCAGCCAG
GAPDH	F	CTGGCCAAGGTCATCCATGAC
	R	CTTGCCCACAGCCTTGGCAG

correlation analysis and found that *NDUFA1* was positively correlated with gamma delta T cells, resting CD4 memory T cells, activated natural killer (NK) cells, and monocytes, and negatively correlated with Tregs, M0 macrophages, resting NK cells, activated mast cells, and naüve CD4 T cells (Fig. 6 C). The relevance between *NDUFS5* and immune cells was almost the same as *NDUFA1*, except that *NDUFS5* not positively correlated with monocytes but was negatively correlated with neutrophils (Fig. 7D). The above results suggested that both candidate MRGs were closely related to immune cell types.

Prediction model was successfully constructed

Age is an important risk factor for the onset of AD. According to U.S. statistical data, the incidence of AD increases sharply with age: 5.0% for people aged 65 to 74 years, 13.1% of people aged 75 to 84, and 33.2% of people aged 85 or older [5]. To further improve the disease prediction accuracy, independent predictors, including age, *NDUFA1*, and *NDUFS5*, were selected to construct the LOAD prediction model, which is presented as a nomogram (Fig. 7A). The AUC of the prediction nomogram was 0.723 with all three factors and 0.708 without age (Fig. 7B). The calibration curve of the LOAD nomogram showed that the overall predicted probability matched the actual probability very well (Fig. 7 C). The DCA for the LOAD nomogram presented that if the threshold probability were over 0.04, using this LOAD nomogram to predict LOAD would bring more benefits than risks for patients (Fig. 7D). Prediction model was successfully constructed and the evaluation indicators were good.

Differential expression of MRGs was verified by qRT-PCR

To further verify the differential expression of the candidate MRGs in LOAD, MCI, and CN, peripheral blood samples were collected from Ruijin Hospital for validation by qRT-PCR. We recruited 9 participants who were CN, 8 patients with LOAD, and 10 patients with MCI. Patient information is shown in Table 4, and all participants were over 65 years old. Their blood samples were collected, and the expression of the three candidate genes was verified by qRT-PCR. The results showed that all three genes, *NDUFA1* (Fig. 8A), *NDUFB3* (Fig. 8B), and *NDUFS5* (Fig. 8 C) had lower expression in patients with LOAD than CN. In addition, *NDUFA1* and *NDUFB3* were significantly decreased in patients with MCI when compared to CN, confirming our conclusions from the public database. The results supported three candidate MRGs as potential diagnostic markers for LOAD and MCI in individuals over 65 years of age.

DISCUSSION

In this study, we found that the differential expression of two MRGs, *NDUFA1* and *NDUFS5*, in peripheral blood can be used as diagnostic markers for patients with LOAD and MCI over 65 years of age. A LOAD diagnosis model was successfully constructed by combining the two candidate MRGs with age. At the same time, changes were found in the mitochondria-related pathways and immune cell composition in the peripheral blood of patients with LOAD.

The pathological mechanisms and etiology of AD remain unclear, and there is a lack of convenient and quick indicators for early screening and diagnosis. Although the main pathological changes of AD occur in the brain, obtaining brain tissue for research purposes is difficult while patients are alive, and few patients with LOAD donate their bodies for scientific research. Therefore, using brain tissue sample indicators as biomarkers for early AD diagnosis is not feasible. Instead, blood-based markers offer a promising, minimally invasive approach for diagnostic purposes. The $A\beta_{42}/A\beta_{40}$ ratio and phosphorylated tau have shown potential as blood-based biomarkers for AD [19]. Plasma $A\beta_{42}/A\beta_{40}$ levels have been demonstrated to predict the status of Aβ deposition in PET-CT. However, the utility of these biomarkers is subject to the variability in detection methods and cohort studies, resulting in varying AUC values ranging from 0.64 to 0.87, mostly between 0.7–0.8 [40–43]. Two large-scale cohort studies reported AUC values of 0.89 versus

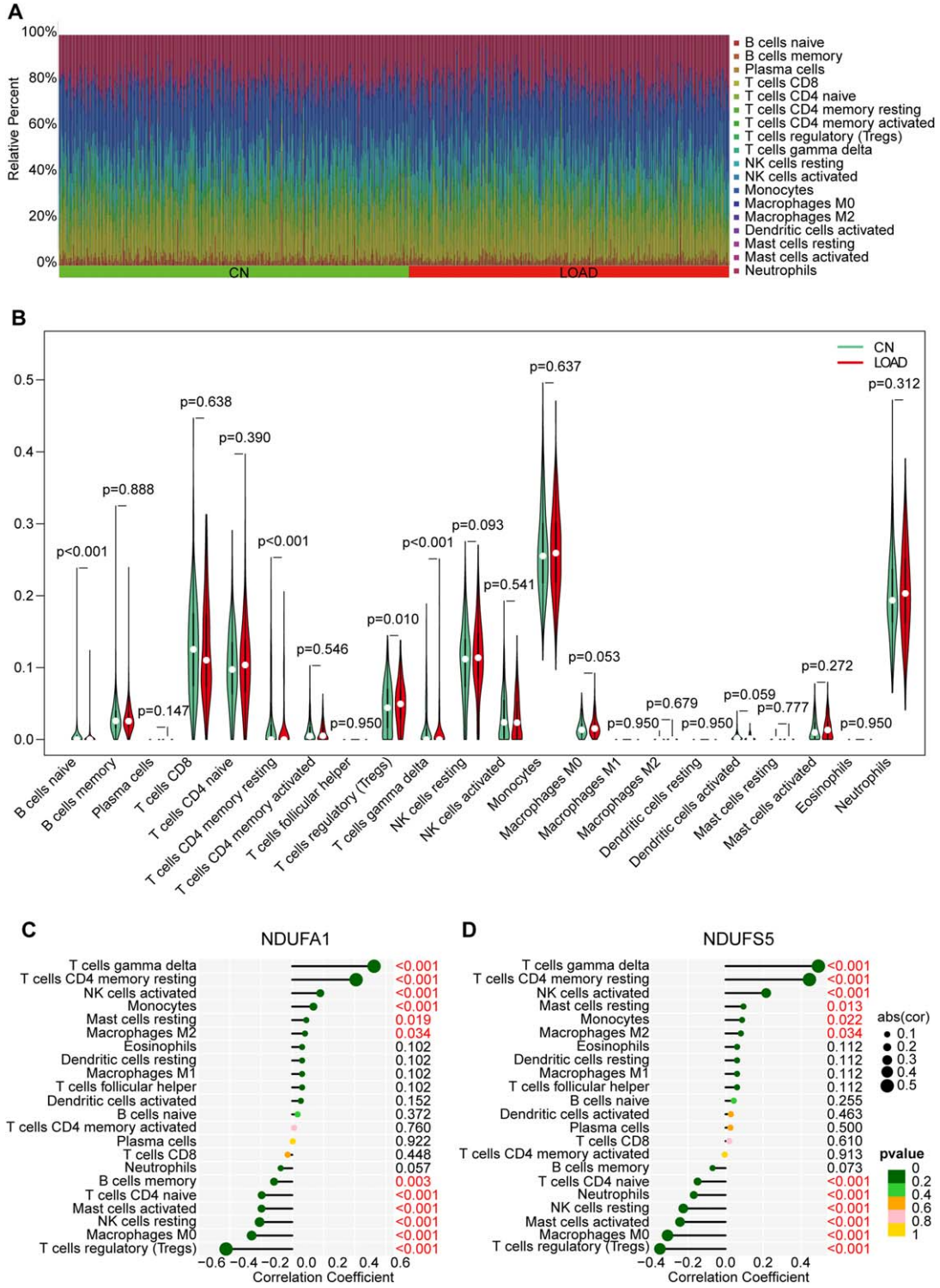

Fig. 6. Immune infiltration between LOAD and CN. A) Relative proportion of peripheral blood infiltrates of 22 distinct subtypes of immune cells in LOAD patients. B) Comparison of 22 immune cell types between CN and LOAD. Green represents normal and red represents LOAD. C, D) The correlation of NDUFA1 (C) and NDUFS5 (D) with immune cells.

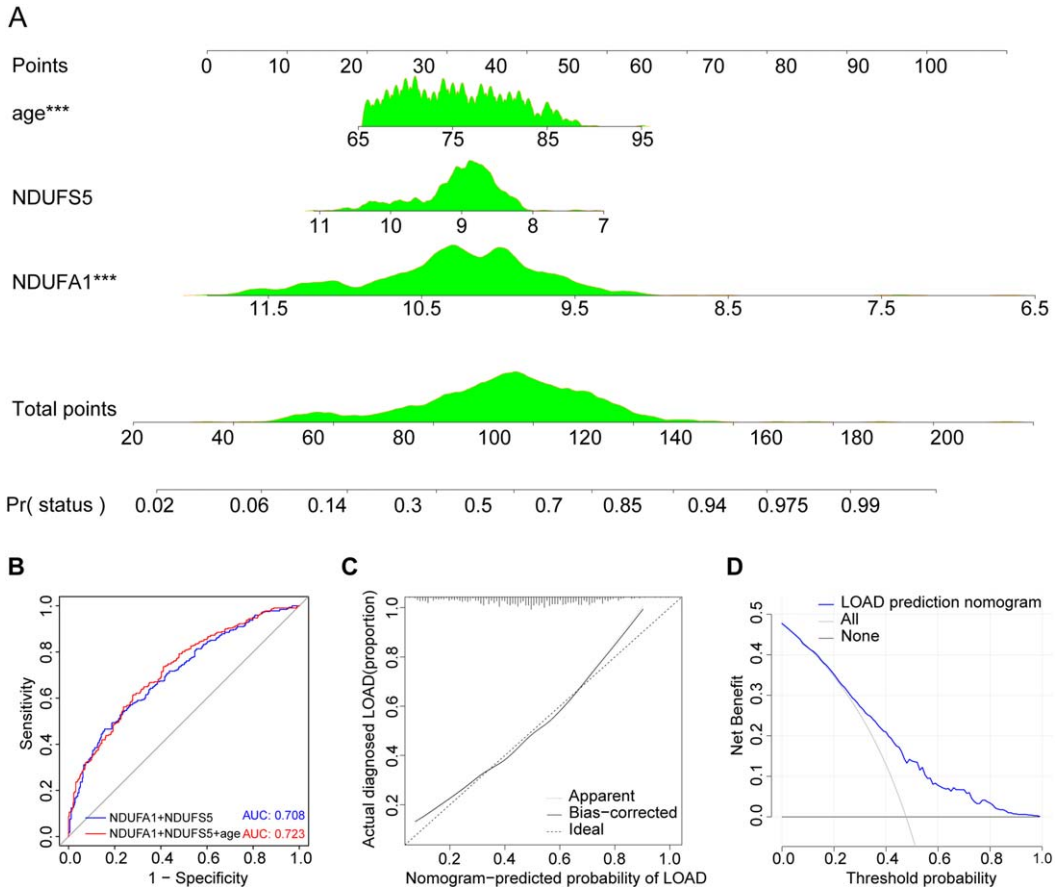

Fig. 7. Model construction and evaluation. A) The LOAD nomogram was established for age and expression of NDUFA1 and NDUFS5 in the cohort. B) ROC curve and corresponding AUC value. C) Calibration curves of the prediction nomogram in the cohort. D) Decision curve analysis for the prediction nomogram.

Table 4
Patients information

	CN	MCI	LOAD	*p*
N	9	10	8	
Age (y, mean ± SD)	72.8 ± 6.6	72.2 ± 4.1	73.4 ± 7.2	0.8189
MMSE score (mean ± SD)	29.8 ± 0.4	27.3 ± 1.8	20.5 ± 3.2	<0.0001
MoCA score (mean ± SD)	28.9 ± 0.9	22.5 ± 3.5	15.83 ± 2.3	<0.0001

MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; SD, standard deviation.

0.72 [44] and 0.83 versus 0.76 [45] for p-tau217 and p-tau181, respectively, in distinguishing between AD versus non-AD. Our approach, based on transcriptome analysis, has an AUC of 0.72 for distinguishing between CN and LOAD, which is comparable to the two classic plasma biomarkers mentioned above. While the detection of plasma Aβ and tau is expensive and subject to measurement variations caused by pre-analytical processing and analytical performance [20], our method offers a simple, practical, and cost-effective alternative that can be applied on a large scale in clinical settings.

Several studies have been conducted to screen DEGs as biomarkers for AD, some of which directly screened DEGs [25, 46], and some focused on the specific fields related to the possible etiology and pathology of AD, such as the immune microenvironment [24], iron metabolism [23], and concomitant diseases [22]. However, most studies did not distinguish between LOAD and EOAD. Compared to

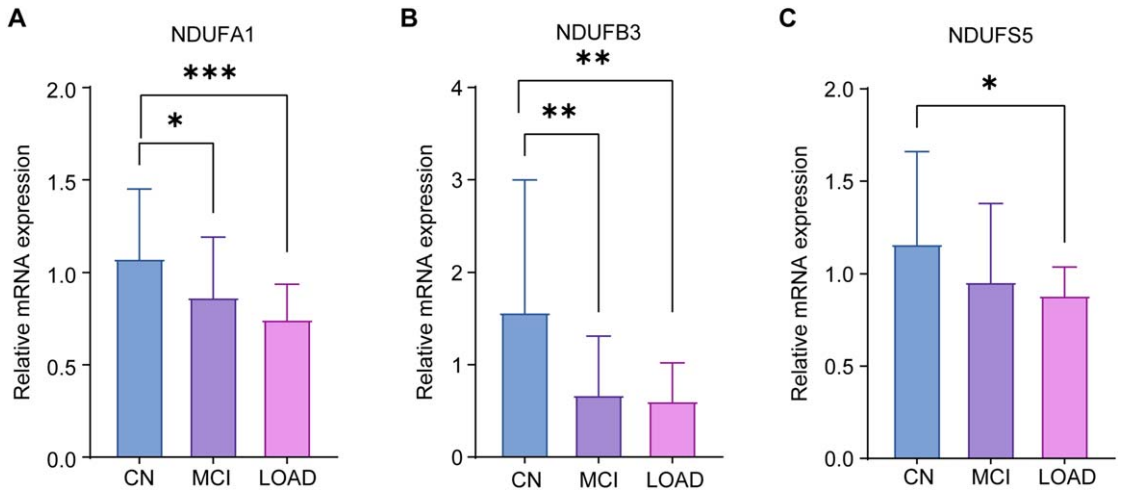

Fig. 8. qRT-PCR validation results. qRT-PCR was used to verify the expression of NDUFA1 (A), NDUFB3 (B), and NDUFS5 (C) in CN, LOAD, and MCI. The experiments were performed in triplicate, and the data were expressed as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, no significance).

LOAD, EOAD has heterogeneous clinical manifestations [47], an aggressive clinical course [48], different pathogenic mechanisms, and different gene changes [49], which may have a confounding effect on research results. At the same time, due to the inclusion of EOAD, there were also some younger individuals in the control group, which could not accurately reflect differences due to LOAD. Therefore, we believe that there is a need for more precise biomarker exploration in the LOAD subgroup. In studies of genetic diagnostic markers for AD, blood and brain tissue samples are often used. There is an interaction between immune cells in the blood and central nervous system [50–52], and numerous studies on neurodegenerative diseases have found that changes in the peripheral blood can indicate the state of the disease to a certain extent [38, 53]. Considering the practicability, simplicity, cost, and availability of the samples, we chose blood samples for this study.

We used bioinformatic analyses to identify gene expression changes in LOAD. Seventy-eight DEGs were identified in the peripheral blood of patients with LOAD. SVM–REF and LASSO algorithms were performed to determine three candidate MRGs as potential biomarkers for LOAD. After validation, *NDUFA1* and *NDUFS5* were selected as the candidate genes for additional analyses. *NDUFA1* is one of the "accessory proteins" identified in complex I [54]. Mitochondrial complex I is the primary entry point for electrons in the electron transport chain and is composed of core proteins and accessory proteins that perform bioenergetic functions [55]. Accessory proteins are not directly involved in catalysis but mainly maintain the structural stability of the complex and play a protective role in the response to oxidative damage [56]. The loss of the *NDUFA1*-encoded protein can cause complex-I deficiency, inhibit caspase activation and apoptosis, and enhance cell death induction [57]. Mutations in *NDUFA1* may play a role in early-onset dementia [58]. *NDUFS5* is also an accessory subunit of mitochondrial complex I [59]. The ND2-module is one of the seven core mtDNA-encoded subunits in mitochondrial complex I [60–63]. ND2 is critical for complex I assembly, the presence of core ND2-module subunits is a necessary condition for the stability of the complex [60]. Once the accessory subunits cannot enter the complex properly, the cell energy loss will increase and a large number of assembly factors will be required to maintain the biological function of complex 1 [64]. The latest assembly stages of the ND2-module of complex I involve the incorporation of subunits *NDUFA1*, *NDUFA10*, and *NDUFS5* [61]. Changes in the expression of *NDUFA1* and *NDUFS5* may affect the assembly of the ND2-module, and thus, the structure and function of mitochondrial complex I.

Our findings on MRGs with decreased expression in LOAD and MCI are practical for clinical application and helpful for the understanding of LOAD pathogenesis, diagnosis and prevention. The cause of AD is not clear at present, but like other chronic degenerative diseases, it may be caused by a variety of complex factors [5]. There are many different theories about the pathogenesis of AD, including

the mitochondrial cascade hypothesis, an important theory considering that mitochondrial dysfunction causes energetic and metabolic dysfunction and also drives the pathogenesis of AD, including Aβ plaque formation and tau deposition [65]. Evidence demonstrates both metabolic defects and oxidative damage occur in AD. Further, a mitochondrial complex I inhibitor restored synaptic activity and cognitive function in 3xTg-AD mice and significantly reduced the levels of pTau [66]. In another study, the mitochondrial function of peripheral blood mononuclear cells and platelets were measured, and the bioenergetic parameters, in descending order, were MCI, CN, and AD. They also found that respiration was positively associated with hippocampal volume, and systemic mitochondrial dysfunction was associated with cognitive decline [67]. It would be intriguing to investigate whether the three markers we identified also contribute to the pathological mechanisms of LOAD in the brain.

The interaction between immune signaling and the intrinsic cellular metabolic program determines the functional state of T lymphocytes [68]. Both mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis are important metabolic pathways that promote T-cell proliferation [69]. In terms of biological energy, resting T cells are characterized by low metabolic requirements, dependence on OXPHOS-derived ATP, and inhibition of glycolysis [70]. Mitochondrial ATP production is essential for T cell activation, and their proliferation is associated with significant glucose uptake and glycolysis, which are the main sources of ATP. Mitochondrial respiration is enhanced by T-cell activation [53, 71]. The expression changes of MRGs in LOAD, such as *NDUFA1*, *NUDFS5*, and *NDUFB3*, may be related to changes in the immune cells in AD peripheral blood, the specific biological processes affected will be explored in our following studies. Our study showed that the LOAD group had significantly higher proportions of Tregs and gamma delta T cells, and lower proportions of resting CD4 memory T cells and naüve B cells. Among the proportion of changed cells, three candidate genes were positively correlated with gamma delta T cells and resting CD4 memory T cells, and negatively correlated with Tregs. Studies on Tregs in AD have been inconsistent. Some studies have revealed that the frequency of Tregs increases with age and is accompanied by intensified suppressive activity of Tregs in patients with AD [39, 72, 73], which is consistent with our results. However, a recent study found that the proportion of circulating Tregs in descending order was MCI, CN, and AD [74], which is inconsistent with our analysis. Differences in results are probably due to different research methods and samples. The CDR3 region of T-cell receptor γ genes in AD brain tissue and peripheral blood is unique. AD brain hydrophilic residues increased, as well as clones with larger volumes [75], which may be related to the inflammatory process of AD. As for the relationship between resting CD4 memory T cells and AD, one study found six kinds of inflammatory cells infiltrating 13 brain regions, and resting CD4 memory T cells had the highest proportion [76]. Some studies suggest that resting CD4 memory T cells may be involved in the AD process [77, 78]. It has also been reported that there is a significant reduction in naive B cells in the peripheral blood of patients with AD [79, 80].

The changes in peripheral blood mitochondrial function found by our enrichment analysis may reflect the dysfunction of brain mitochondria in patients with LOAD to a certain extent, and the specific correlation and mechanism need to be further explored. Peripheral circulating immune cells may have crosstalk with the central nervous system (CNS). Immune cells in the peripheral blood also exist in the CNS, and immune surveillance through the selected peripheral white blood cells provides a maintenance mechanism that is essential for brain function [81]. Episodes in neurodegenerative diseases occur when the presence of pathological mediators in the CNS overrides this capacity for immune surveillance [82]. Along the gut-brain axis, Tregs interact with a variety of resident cells in the CNS, including immune, epithelial, and neuronal cells, to produce a powerful neuroprotective effect in neuronal diseases [50, 51]. It has also been shown that *Chlamydia pneumoniae* infection may lead to dysregulation of key pathways involved in AD pathogenesis after intranasal inoculation [83]. Moreover, circulating blood cells are exposed to paracrine factors that regulate mitochondrial function throughout the body, possessing high ETC activity and metabolic flexibility [84], and have long been considered as a potential sensitive marker of mitochondrial dysfunction [85]. Blood cell bioenergetics can indicate the bioenergetics of high metabolically active tissues such as brain [86].

AD is a neurodegenerative disease with insidious onset and gradual development [2]. Pathological changes such as tau protein deposition occur before clinical symptoms [15], and mitochondrial dysfunction in the brain can be detected in the MCI stage [87]. We were curious whether the MRG alterations

identified in this study occurred in the MCI stage, so we validated them on the gene datasets and collected clinical peripheral blood samples. Dataset analysis showed that *NDUFA1* and *NDUFS5* were significantly decreased in patients with MCI compared to the CN group, and both had good prediction accuracy for MCI. The two candidate genes we identified can predict LOAD earlier and provide help for early detection and intervention of LOAD.

We performed qRT-PCR validation on clinical peripheral blood samples, and found that *NDUFA1*, *NDUFS5*, and *NDUFB3* were significantly decreased in LOAD compared to CN. *NDUFA1* and *NDUFB3*, but not *NDUFS5*, were significantly decreased in patients with MCI compared to CN. LOAD exhibited more significant changes in MRGs. This may be related to the different sources and scales of patients between the clinical samples and datasets, and the reason needs to be further explored.

Although we identified some MRGs that can serve as candidate genes for LOAD and MCI using bioinformatics methods and qRT-PCR experiments, our study still has limitations. First, the clinical validation sample size in this study was small and came from a single center, therefore, the conclusion may lack the universality of other regions and populations. In the future, more samples should be collected to verify the correlation between MRGs and LOAD. Second, the experiments in this study did not classify peripheral blood leukocytes, subsequent studies could classify leukocytes to explore specific cell groups with significant changes in MRGs expression. Third, the molecular biological mechanisms between down regulated MRGs and LOAD needs to be further explored, which will be shown in our following work.

Conclusion

Using the GEO public database and machine learning methods, including LASSO and SVM-RFE, we identified two MRGs, *NDUFA1* and *NDUFS5*, which can be used as candidate genes of MCI and LOAD, and we constructed a disease prediction model. The results were verified by qRT-PCR of clinical blood samples. Biological function analysis showed that the expression of mitochondria-related pathways was significantly changed. This study also reported changes in LOAD peripheral circulating immune cells, and Tregs and resting CD4 memory T cells were closely related to changes in candidate genes, the specific mechanism will be further explored.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article material. The original data supporting this study can be obtained by contacting the corresponding author.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: https://dx.doi.org/10.3233/JAD-230314.

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