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ORIGINAL RESEARCH

Serum Lipid Biomarkers for the Diagnosis and Monitoring of Neuromyelitis Optica Spectrum Disorder: Towards Improved Clinical Management

Ruibing Li[1,*], Jinyang Wang[1,2,*], Jianan Wang[1,*], Wei Xie[3], Pengfei Song[4], Jie Zhang[4], Yun Xu[5], Decai Tian[5], Lei Wu[3,*], Chengbin Wang[1,*]

[1]Department of Laboratory Medicine, the First Medical Centre of Chinese PLA General Hospital, Beijing, 100853, People's Republic of China; [2]School of Laboratory Medicine, Weifang Medical College, Weifang, Shandong, 261053, People's Republic of China; [3]Department of Neurology, the First Medical Centre of Chinese PLA General Hospital, Beijing, 100853, People's Republic of China; [4]School of Advanced Technology, Xi'an Jiaotong - Liverpool University, Suzhou, 215000, People's Republic of China; [5]Center for Neurology, Beijing Tiantan Hospital, Capital Medical University, Beijing, 100050, People's Republic of China

*These authors contributed equally to this work

Correspondence: Lei Wu; Chengbin Wang, The First Medical Centre of Chinese PLA General Hospital, Beijing, 100853, People's Republic of China, Email wlyingsh@163.com; wangcb301@126.com

Background: Neuromyelitis optica spectrum disorder (NMOSD) is a group of immune-mediated disorders that often lead to severe disability. The diagnosis and monitoring of NMOSD can be challenging, particularly in seronegative cases, highlighting the need for reliable biomarkers to enhance clinical management. This study aimed to identify serum lipid biomarkers for the diagnosis and monitoring of NMOSD and to assess their potential to improve clinical decision-making.

Methods: We conducted a comprehensive serum proteomic analysis in a discovery cohort of NMOSD patients and controls to identify lipid-related proteins associated with NMOSD. Subsequently, we validated the candidate biomarkers in the retrospective cohort and developed diagnostic models using a random forest algorithm. The association between these lipid biomarkers and disease activity was further evaluated in longitudinal analysis.

Results: Our analysis identified a panel of serum lipid-related biomarkers that demonstrated significant differences between NMOSD patients and controls. The diagnostic models achieved the impressive accuracy of 72% for the full NMOSD spectrum, 72% for AQP4-IgG+ NMOSD, and 68% for double seronegative NMOSD. Importantly, these biomarkers showed a correlation with disease activity, with levels changing from relapse to remission. Additionally, a combination of these lipid biomarkers was found to predict relapse with the AUC of 0.861. A user-friendly smartphone application was developed to facilitate the straightforward "input-index, output-answer" screening process, enhancing both clinical decision-making and patient care.

Conclusion: The diagnostic model based on the serum lipid-related indexes (TC, TG, LDL, HDL, ApoA1, and ApoB) may be the useful tool for NMOSD in diagnosis and monitoring of disease stage, thereby improving the treatment outcome for patients. Future studies should focus on integrating these biomarkers into routine clinical practice to realize their full potential in enhancing NMOSD management.

Keywords: Neuromyelitis optica spectrum disorder, serum proteomic profiles, serum lipid-related indexes, diagnosis biomarker, disease monitoring

Introduction

Neuromyelitis optica spectrum disorder (NMOSD) is a group of autoimmune demyelinating conditions of central nervous system (CNS), which mainly involves the spinal cord and the optic nerve. Clinically, NMOSD has multiple symptoms similar to multiple sclerosis (MS), MOG-IgG associated disorders (MOGAD) or other inflammatory demyelinating diseases, which impedes the differential diagnosis.[1–3] Misdiagnosis and missed diagnosis of this disease would

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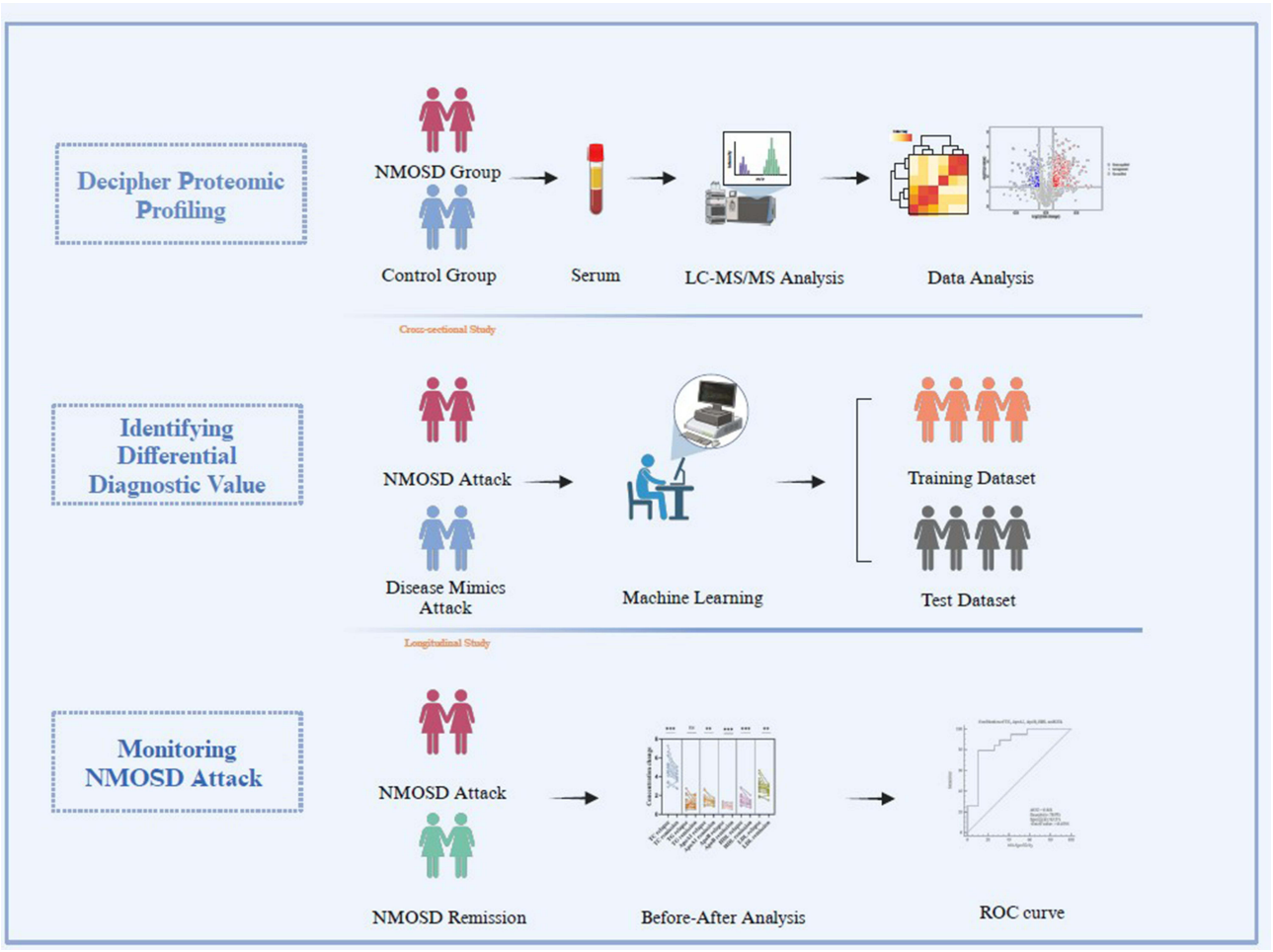
Graphical Abstract



inevitably lead to delayed treatment and inappropriate therapeutic regimens. For patients with NMOSD, the therapeutic regimen of MS may aggravate their conditions.[4] The successive discovery of specific antibodies aquaporin-4 (AQP4)-IgG[5,6] and myelin oligodendrocyte glycoprotein (MOG)-IgG[2,7] contributes to precise diagnosis of NMOSD, MS and MOGAD,[1,3,8] while AQP-4 and MOG antibodies can not reflect disease activity and severity. Additionally, for those NMOSD patients with atypical clinical features or double seronegative NMOSD (including inaccessibility of the antibody detection) in the early stage of the disease, there is an urgent need for effective biomarkers. Therefore, biomarkers for disease diagnosis and monitoring are of great significance for NMOSD, especially for double seronegative NMOSD.[8,9]

Along with the continuous deepening of research on biomarkers, emerging novel markers such as glial fibrillary acidic protein (GFAP) and neurofilament light chain (NfL) have been reported,[10,11] which improved the diagnosis and progression monitoring of NMOSD to some extent. Our previous study[12] deeply mined and validated that sGFAP, sNfL, sUCHL1 levels were elevated in NMOSD patients with relapse, and significantly associated with the major lesion of the patients, which can be novel candidates for early diagnosis. However, due to limitations such as novel biomarkers' extremely low concentration in blood, the lack of widespread clinical application of ultrasensitive biochemistry analyzer, and high costs, routine testing for GFAP and NfL in blood has not yet been achieved in all hospitals, especially in primary hospitals.[8]

Among the potential candidates, serum lipid biomarkers have garnered considerable attention. Lipids are not only crucial for cellular structure and function but also play a significant role in immune regulation and inflammation.[13–15] Emerging evidence suggests[16,17] that lipid metabolism is disrupted in NMOSD, offering a novel avenue for biomarker research. Importantly, serum lipids have been included in clinical routine biochemical tests and extensively carried out to monitor lipid changes in the setting of various diseases. Therefore, considering the high abundance of blood lipids and the convenience of clinical lipid testing, we proposed lipids as candidate biomarkers and constructed diagnostic models of NMOSD.

In summary, this study aims to explore novel protein markers in blood samples for NMOSD through proteomics, and to analyze their efficacy in terms of diagnosis and relapse monitoring of the full NMOSD spectrum (especially double seronegative NMOSD). In addition, our study also provides new insights into the pathogenesis of NMOSD.

Materials and Methods

Cohort Design, Setting and Participants

This study consisted of a discovery cohort and validation cohorts, as outlined in Figure 1. Patients were recruited from the First Medical Center of Chinese PLA General Hospital and diagnosed by experienced neurologists according to international diagnostic criteria of corresponding neurological disorders.[1,18] Demographic characteristics of the candidates are listed in Table 1. Following the definitions set out in Ma et al,[19] patients diagnosed with NMOSD and MS were divided into attack and remission stages. The attack stage was defined as the appearance of new clinical manifestations and the aggravation of original symptoms. And remission was defined as symptoms relieved for over one month and the condition remained stable. Patients with fever, hypertension, infectious diseases, autoimmune diseases, metabolic disease, tumors, pregnant and other nervous system damages were excluded. All of participants were not involved in disease modifying treatment (DMT) exposure. Specific antibody detection used cell-based transfection

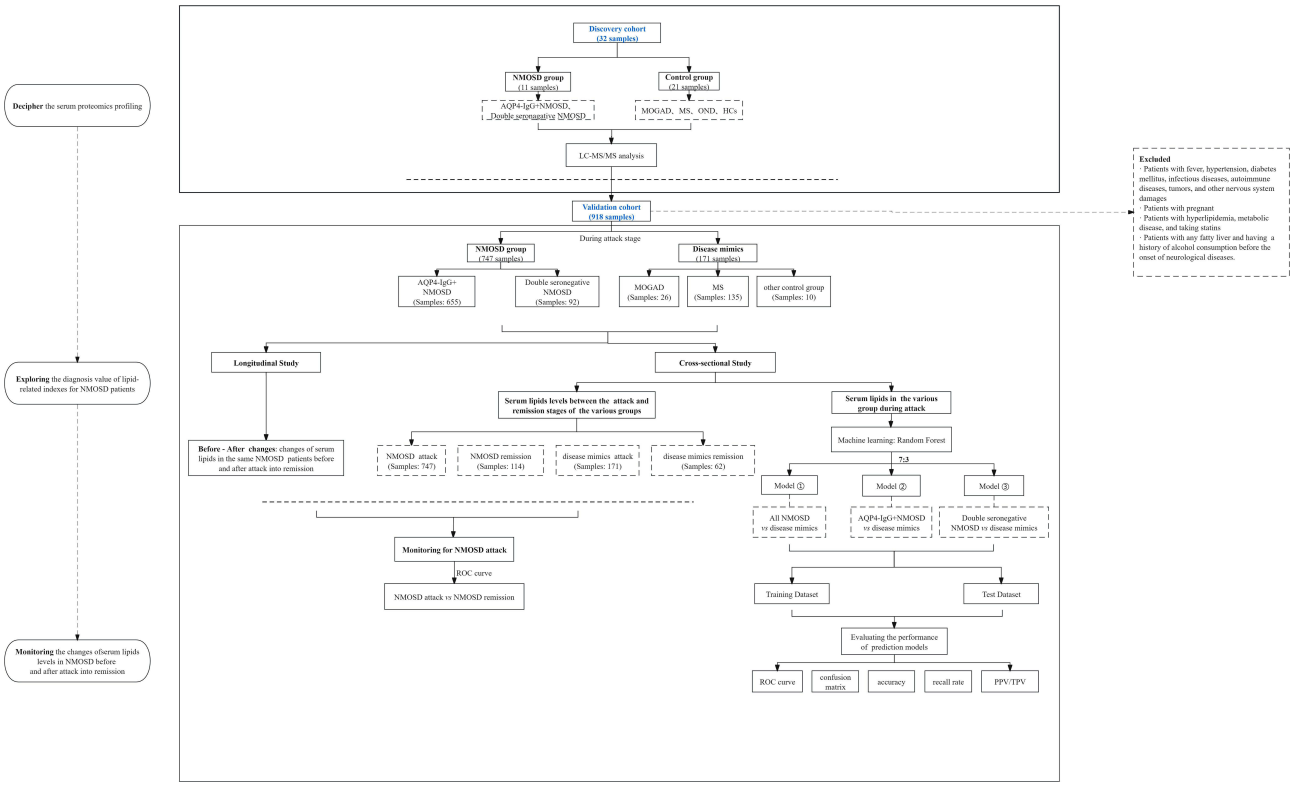


Figure 1 Overall design of the study.

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Table 1 Demographic and Clinical Data of the Discovery Cohort

	Discovery cohort (n = 32)					
	NMOSD (n = 11)			Control (n = 21)		
	AQP4+ (n =9)	Double seronegative (n =2)	MS (n = 6)	MOGAD (n = 3)	OND (n = 2)	HC (n = 10)
Age, year, s	44.0±6.0	43±7.5	33±2.0	36±3.6	33±8.5	48±5
Female, n (%)	9 (100.00%)	0	6 (100.00%)	3 (100.00%)	1 (50.00%)	10 (100.00%)
Disease duration, [m, ±s]	72.5±39.1	3.0±1.0	87.0±18.6	10.7±0.9	2.5±1.5	/
BMI / (kg/m^2), ±s	24.7±1.3	29.5±0.4	18.4±0.7	25.6±2.8	23.0±4.7	
Lesion in MRI during recent attack, n (%)						
Only Optic nerve	3 (42.86%)	1 (50.00%)	1 (16.67%)	0	1 (50.00%)	/
Only Spinal cord	0	1 (50.00%)	0	1 (33.33%)	1 (50.00%)	/
Only Brain	1 (14.29%)	0	1 (16.67%)	1 (33.33%)	0	/
Optic nerve +Spinal cord	1 (14.29%)	0	0	0	0	/
Optic nerve +Brain	0	0	0	0	0	/
Spinal cord +Brain	2(28.57%)	0	4 (66.67%)	1 (33.33%)	0	/
Optic nerve +Spinal cord+Brain	0	0	0	0	0	/

Notes: In the discovery cohort, NMOSD included AQP4-IgG+NMOSD and double seronegative NMOSD, and control group included MOGAD, MS, other neurological disease (OND, including optic neuritis and myelitis) and healthy controls (HC).

Abbreviations: NMOSD, neuromyelitis optica spectrum disorder; AQP4, aquaporin-4; AQP4-IgG+NMOSD, AQP4 antibody positive NMOSD; MOG, myelin oligodendrocyte glycoprotein; MOGAD, MOG-antibody-associated disease; MS, multiple sclerosis; ALS, amyotrophic lateral sclerosis; BMI, body mass index; IQR, inter quartile range; M, median; y, year; m, month; n, number; N, total number; %, percentage.

immunofluorescence assay (CBA). Our study complied with the Declaration of Helsinki and was approved by Ethics Committee of Chinese PLA General Hospital (No. S2021-128-01, 02).

Discovery cohort: A total of 32 samples were enrolled for differential proteomic analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to screen out potential biomarkers, including 11 NMOSD patients and 21 Control participants (detailed in Table 1).

Validation cohorts: Multiple longitudinal and cross-sectional cohorts were established to validate the value of potential biomarkers in the diagnosis of the full NMOSD spectrum. The cohort used to construct diagnostic models consisted of patients in the attack stage from January 1, 2015, to December 31, 2022, and a total of 918 serum lipid profiles were retrospectively collected from medical records, including 747 NMOSD samples (from 626 patients), 26 MOGAD samples (from 21 patients), 135 MS samples (from 109 patients) and 10 other differential controls (optic neuritis, myelitis and ALS). The detailed information was listed in Table 2. Subsequently, remission cases from the same period were included to assess the relationship between lipid metabolism and disease stages.

Sample Collection and Preparation

Fasting serum samples were collected on admission within 24 hours. These samples were then aliquoted and preserved at −80 °C. Measurement of serum samples was performed by investigators who were blinded to clinical data.

For LC-MS/MS analysis in the discovery cohort, the samples were prepared with serum denaturation, disulfide reduction, cysteine alkylation, buffer exchange, trypsin digestion, and desalting prior to analysis to produce peptides. The prepared peptides were separated and identified by liquid chromatography (Ultimate 3000, Thermo Fisher) Tandem Mass Spectrometry (Q-Exactive, Thermo Fisher, USA) in Data-dependent acquisition (DDA) mode.

For the validation cohorts, total cholesterol (TC) and triglycerides (TG) were measured using enzymatic colorimetry assay, and Apolipoprotein A1 (ApoA1), Apolipoprotein B (ApoB), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were measured using immunoturbidimetry on Roche Cobas®800 automatic biochemical analyzer with quality control.

Table 2 Demographic and Clinical Data of Validation Cohorts During Attack

Validation Cohorts (n = 918)	NMOSD (n = 747)	Disease Mimics (n = 171)		
		MOGAD (n= 26)	MS (n = 135)	Other Control Group (n = 9)
Age, y, M (IQR)	39 (28, 52)	29 (21, 33)[A]	32 (27, 45)[A]	51 (26, 55)
Female sex,n (%)	668 (89.42%)	18 (69.23%)[a]	80 (59.26%)[a]	7 (70.00%)
NMOSD patients during recent attack, n (%)				
AQP4 antibody positive	655 (87.68%)	/	/	/
Double seronegative	92 (12.32%)	/	/	/
Disease duration, [m, M (IQR)]	24.00 (4.00, 60.00)	8.0 (2.25, 24.00)[A,B]	32.00 (6.00, 84.00)	1.00 (1.00, 4.00)[B]
BMI (kg/m^2),[M (IQR)]	22.70 (20.31, 25.20)	23.35 (21.20, 25.60)	22.40 (20.20, 24.80)	21.50 (18.30, 26.30)
Lesion in MRI during recent attack, n (%)				
Only Optic nerve	401 (53.68%)	19 (73.08%)	2 (1.48%)[a]	5 (50.00%)
Only Spinal cord	147 (19.68%)	2 (7.69%)	17 (12.59%)	4 (40.00%)
Only Brain	15 (2.01%)	1 (3.85%)	44 (32.59%)[a]	1 (10.00%)
Optic nerve +Spinal cord	105 (14.06%)	1 (3.85%)	2 (1.48%)[a]	0
Optic nerve +Brain	13 (1.74%)	1 (3.85%)	4 (2.96%)	0
Spinal cord +Brain	47 (6.29%)	2 (7.69%)	62 (45.93%)[a]	0
Optic nerve +Spinal cord+Brain	19 (2.54%)	0	4 (2.96%)	0

Notes: The data for this validation part were all collected from patients in the attack stage. NMOSD group included AQP4-IgG+NMOSD and double seronegative NMOSD, and disease mimics included MOGAD, MS, other neurological disease (including optic neuritis and myelitis, ALS). Multiple comparisons of *p*-values were corrected using the Bonferroni method. vs NMOSD: [A], $p < 0.01$; vs MS: [B] $p < 0.01$; For categorical data, vs NMOSD: [a], $p < 0.05$.

Abbreviations: NMOSD, neuromyelitis optica spectrum disorder; AQP4, aquaporin-4; AQP4-IgG+NMOSD, AQP4 antibody positive NMOSD; MOG, myelin oligodendrocyte glycoprotein; MOGAD, MOG-antibody-associated disease; MS, multiple sclerosis; ALS, amyotrophic lateral sclerosis; BMI, body mass index; IQR, inter quartile range; M, median; y, year; m, month; n, number; N, total number; %, percentage.

Bioinformatics Analysis

MS raw data were searched against the human UniProt database using Sequest HT through Proteome Discoverer and processed with Perseus software (version 1.6.15.0). The protein abundance data were annotated, filtered by valid values (at least >50% valid values in each group per protein), and binary logarithmic transformed. After missing value replacement, the data were normalized, the differential protein abundances between groups were analyzed with two tails Welch's *t* test. And proteins with $p < 0.05$ were considered statistically different. Enrichment analysis was conducted to interpret the relevant biological functions (Hiplot, https://hiplot.com.cn; MetaboAnalyst, https://www.metaboanalyst.ca/faces/home.xhtml; WikiPathways, https://www.wikipathways.org; OECloud, https://cloud.oebiotech.cn).

Machine Learning - Random Forests

Based on lipoproteins' difference between groups in discovery cohort, the retrospective cohort focus on lipids and lipoproteins change were constructed. According to clinical problems, the retrospective validation cohort was divided into three datasets to build diagnostic models for patients in the attack stage with NMOSD (model 1), AQP4-IgG+ NMOSD (model 2), and double seronegative NMOSD (model 3), respectively (Figure 1).

The age, TC, TG, ApoA1, ApoB, HDL and LDL of patients as variables were brought into six machine learning classifiers, including Logistic Regression (LR), K-Nearest Neighbor (KNN), Support Vector Machine (SVM), Decision Tree (DT), Random Forest (RF), and Neural Network. The Random Forest with highest accuracy values across all models was selected for diagnostic model building. The original data were split into training and test subsets (7:3 ratio of training:test), which were used to train the model and test the predictive ability, respectively. The confusion matrix and the learning curve for the models were built and the efficiency of the classifiers were validated by the sensitivity, specificity, accuracy, precision, recall, F1-score, and AUC-ROC curve calculated using confusion matrix.

Statistics Analysis

Descriptive statistics were performed to summarize the distribution of the continuous (counts, mean ± standard deviation, median ± interquartile range (IQR)) and categorical variables (counts, percentages (%)). Corresponding *t*-test (or *Mann–Whitney* test, *Kruskal–Wallis* test), and *chi-square* test were conducted for different types of variables to compare differences between groups. Multiple comparisons were adjusted with the Bonferroni method. BMI was grouped into underweight (< 18 kg/m^2); normal (18 ~ 25 kg/m^2) and overweight (> 25 kg/m^2). The risk of relapse probability across three BMI groups was plotted and estimated using the Cox Proportional Hazards Model analysis. The model with relapse time interval as the timescale was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) after adjustment of age at relapse and sex. Then, under the normal BMI, the changes of lipid-related variables from relapse to remission stage were analyzed through before - after method, and the variables with significant changes from relapse to remission were selected to built combined ROC curves using the predicted probability of these variables based on the logistic regression model. All statistical analyses were conducted by SPSS 25.0 (IBM Corp., N.Y., USA), GraphPad Prism 8 (SanDiego, CA, USA), and MedCalc 19.0 (Medcalc Software, Ostend, Belgium). A p-value < 0.05 was considered statistically significant.

Results

Demographic Characteristics of the Whole Study

Overall, a total of 950 samples were included in this study. The discovery cohort consisted of 32 samples, while the validation cohorts comprised 918 samples. The demographic data and clinical characteristics were summarized in Tables 1–5.

The discovery cohort was divided into the NMOSD group (AQP4-IgG+NMOSD and double seronegative NMOSD) and Control group (MOGAD, MS, OND and HCs). The data revealed a predominance of females in both

Table 3 Demographic and Clinical Data of Model 1

Model 1	Training Cohort (n = 642)		Test Cohort (n= 276)	
	All NMOSD (n = 526)	Disease Mimics (n = 116)	All NMOSD (n = 221)	Disease Mimics (n = 55)
Age, year, M (IQR)	38 (27, 51)	31 (27, 41)	42 (29, 53)	36 (25, 46)
Female sex, n (%)	472 (89.73%)	71 (61.21%)	196 (88.69%)	34 (61.82%)
NMOSD patients, n (%)				
AQP4 antibody positive	466 (88.59%)	/	191 (86.43%)	/
Double seronegative	60 (11.41%)	/	30 (10.57%)	/
Disease duration, [m, M (IQR)]	24.00 (4.00, 60.00)	19.00 (2.25, 60.00)	24.00 (5.00, 84.00)	32.00 (5.00, 72.00)
BMI (kg/m^2), [M (IQR)]	22.60 (20.30, 25.00)	22.45 (20.30, 24.98)	22.90 (20.70, 25.69)	21.90 (19.00, 24.40)
Phase of attack, n (%)	526 (100%)	116 (100%)	221 (100%)	55 (100%)
Lesion in MRI during recent attack, n (%)				
Only Optic nerve	289 (54.94%)	20 (17.24%)	113 (51.13%)	6 (10.91%)
Only Spinal cord	107 (20.34%)	14 (12.07%)	41 (18.55%)	8 (14.55%)
Only Brain	11 (2.09%)	32 (27.59%)	3 (1.36%)	13 (23.64%)
Optic nerve +Spinal cord	65 (12.36%)	3 (2.59%)	40 (18.10%)	2 (3.64%)
Optic nerve +Brain	9 (1.71%)	4 (3.45%)	4 (1.81%)	1 (1.82%)
Spinal cord +Brain	32 (6.08%)	39 (33.62%)	14 (6.33%)	24 (43.64%)
Optic nerve +Spinal cord+Brain	13 (2.47%)	4 (3.45%)	6 (2.71%)	1 (1.82%)

Notes: According to clinical purpose, data for the validation part were used to build three diagnostic model for attack-stage of NMOSD (model 1), AQP4-IgG+NMOSD (model 2) and double seronegative NMOSD (model 3) using random forest algorithm. In each model, the data were split into training set and test set (7:3 ratio of training: test). Model 1 aimed at distinguishing all NMOSD from disease mimics.

Abbreviations: NMOSD, neuromyelitis optica spectrum disorder; AQP4+, aquaporin-4; AQP4-IgG+NMOSD, AQP4 antibody positive NMOSD; BMI, body mass index; IQR, interquartile range; M, median; y, year; m, month; n, number; N, total number; %, percentage.

Table 4 Demographic and Clinical Data of Model 2

Model 2	Training Cohort (n = 578)		Test Cohort (n= 248)	
	AQP4+ (n = 455)	Disease Mimics (n = 123)	AQP4+ (n = 200)	Disease Mimics (n = 48)
Age, year, M (IQR)	36 (27, 50)	32 (27, 45)	39 (27, 52)	30 (24, 37)
Female, n (%)	423 (92.97%)	77 (62.60%)	175 (87.50%)	28 (58.33%)
NMOSD patients, n (%)				
AQP4 antibody positive	455 (100%)	/	200 (100%)	/
Double seronegative	0	/	0	/
Disease duration, [m, M (IQR)]	24.00 (5.00, 60.00)	24.00 (3.00, 72.00)	24.00 (4.50, 84.00)	24.00 (3.50 61.25)
BMI (kg/m^2), [m, M (IQR)]	22.40 (20.10, 24.61)	22.85 (20.23, 25.63)	22.90 (20.80, 25.80)	21.80 (20.15, 24.05)
Phase of attack, n (%)	455 (100%)	123 (100%)	200 (100%)	48 (100%)
Lesion in MRI during recent attack, n (%)				
Only Optic nerve	274 (60.22%)	17 (13.82%)	110 (55.00%)	9 (18.75%)
Only Spinal cord	76 (16.70%)	16 (13.01%)	39 (19.50%)	6 (12.50%)
Only Brain	7 (1.54%)	31 (25.20%)	4 (2.00%)	14 (29.17%)
Optic nerve +Spinal cord	53 (11.65%)	2 (1.63%)	27 (13.50%)	2 (4.17%)
Optic nerve +Brain	7 (1.54%)	5 (4.07%)	6 (3.00%)	1 (2.08%)
Spinal cord +Brain	23 (5.05%)	48 (39.02%)	13 (6.50%)	15 (31.25%)
Optic nerve +Spinal cord+Brain	15 (3.30%)	4 (3.25%)	1 (0.50%)	1 (2.08%)

Notes: According to clinical purpose, data for the validation part were used to build three diagnostic model for attack-stage of NMOSD (model 1), AQP4-IgG +NMOSD (model 2) and double seronegative NMOSD (model 3) using random forest algorithm. In each model, the data were split into training set and test set (7:3 ratio of training:test). Model 2 focused on distinguishing AQP4-IgG+NMOSD from disease mimics.

Abbreviations: NMOSD, neuromyelitis optica spectrum disorder; AQP4+, aquaporin-4; AQP4-IgG+NMOSD, AQP4 antibody positive NMOSD; BMI, body mass index; IQR, interquartile range; M, median; y, year; m, month; n, number; N, total number; %, percentage.

Table 5 Demographic and Clinical Data of Model 3

Model 3	Training cohort (n = 184)		Test cohort (n= 79)	
	NMOSD (n = 60)	Disease mimics (n = 124)	NMOSD (n = 32)	Disease mimics (n = 47)
Age, year, M (IQR)	44 (33, 52)	32 (27, 43)	55 (33, 64)	30 (25, 45)
Female, n (%)	46 (76.67%)	73 (58.87%)	24 (75.00%)	32 (68.09%)
NMOSD patients, n (%)				
AQP4 antibody positive	0	/	0	/
Double seronegative	60 (100%)	/	32 (100%)	/
Disease duration, [m, M (IQR)]	24.00 (3.00, 90.00)	24.00 (3.00, 66.75)	2.5 (1.0, 42.0)	16.5 (2.0, 81.0)
BMI (kg/m^2), [m, M (IQR)]	23.53 (21.02, 26.11)	22.25 (20.20, 24.80)	24.09 (21.7, 26.1)	23.2 (20.1, 26.4)
Lesion in MRI during recent attack, n (%)				
Only Optic nerve	16 (26.67%)	19 (15.32%)	3 (9.38%)	7 (14.89%)
Only Spinal cord	15 (25.00%)	15 (12.10%)	18 (56.25%)	8 (17.02%)
Only Brain	3 (5.00%)	38 (30.65%)	1 (3.13%)	8 (17.02%)
Optic nerve +Spinal cord	15 (25.00%)	2 (1.61%)	7 (21.88%)	1 (2.13%)
Optic nerve +Brain	0	3 (2.42%)	0	2 (4.26%)
Spinal cord +Brain	9 (15.00%)	44 (35.48%)	2 (6.25%)	20 (42.55%)
Optic nerve +Spinal cord+Brain	2 (3.33%)	3 (2.42%)	1 (3.13%)	1 (2.13%)

Notes: According to clinical purpose, data for the validation part were used to build three diagnostic model for attack-stage of NMOSD (model 1), AQP4-IgG +NMOSD (model 2) and double seronegative NMOSD (model 3) using random forest algorithm. In each model, the data were split into training set and test set (7:3 ratio of training:test). Model 3 focused on distinguishing antibody negative NMOSD from disease mimics.

Abbreviations: NMOSD, neuromyelitis optica spectrum disorder; AQP4+, aquaporin-4; AQP4-IgG+NMOSD, AQP4 antibody positive NMOSD; BMI, body mass index; IQR, interquartile range; M, median; y, year; m, month; n, number; N, total number; %, percentage.

groups, consistent with the current epidemiology of NMOSD. No statistically significant differences were observed between the two groups.

Identification of Serum Lipid Biomarkers in NMOSD Discovery Cohort

Quantitative proteomics analysis from 32 samples of the discovery cohort provided relative abundance data of 343 proteins, of which 57 proteins expressed significantly different ($p < 0.05$ and |FC| >1.5, Figure 2A–C) in NMOSD vs Control. The differentially expressed proteins (DEPs) were composed of 25 proteins significantly up-regulated in NMOSD group and 32 down-regulated proteins. The results of heatmap and principal component analysis provided clear evidence of distinct separation between groups (Figure 2B and C). The results revealed that ApoB and ApoC4 were up-regulated in the NMOSD group (FC: ApoB- 1.5, ApoC4- 2.1, $p < 0.05$). Additionally, ApoE and ApoD showed an upward trend, although the difference was not statistically significant (FC: ApoE- 1.4, ApoD- 1.5, $p > 0.05$) (Figure 2A and C). These findings suggested that apolipoproteins might play a crucial role in the NMOSD.

Furthermore, GO enrichment indicated that the DEPs were involved in processes such as complement activation, chronic inflammation response, very-low-density lipoprotein (VLDL) particle assembly, astrocyte development and maintenance of blood-brain barrier (Figure 2D), which were closely associated with inflammation response and lipids regulation. Pathway analysis further highlighted the high enrichment score for VLDL clearance, suggesting the involvement of lipids in the NMOSD mechanism. Moreover, lipid-related indexes have already been widely available in clinic, which facilitated conducting clinical studies and subsequent analysis. Therefore, we specifically focused on evaluating the lipid-related indexes in the following study.

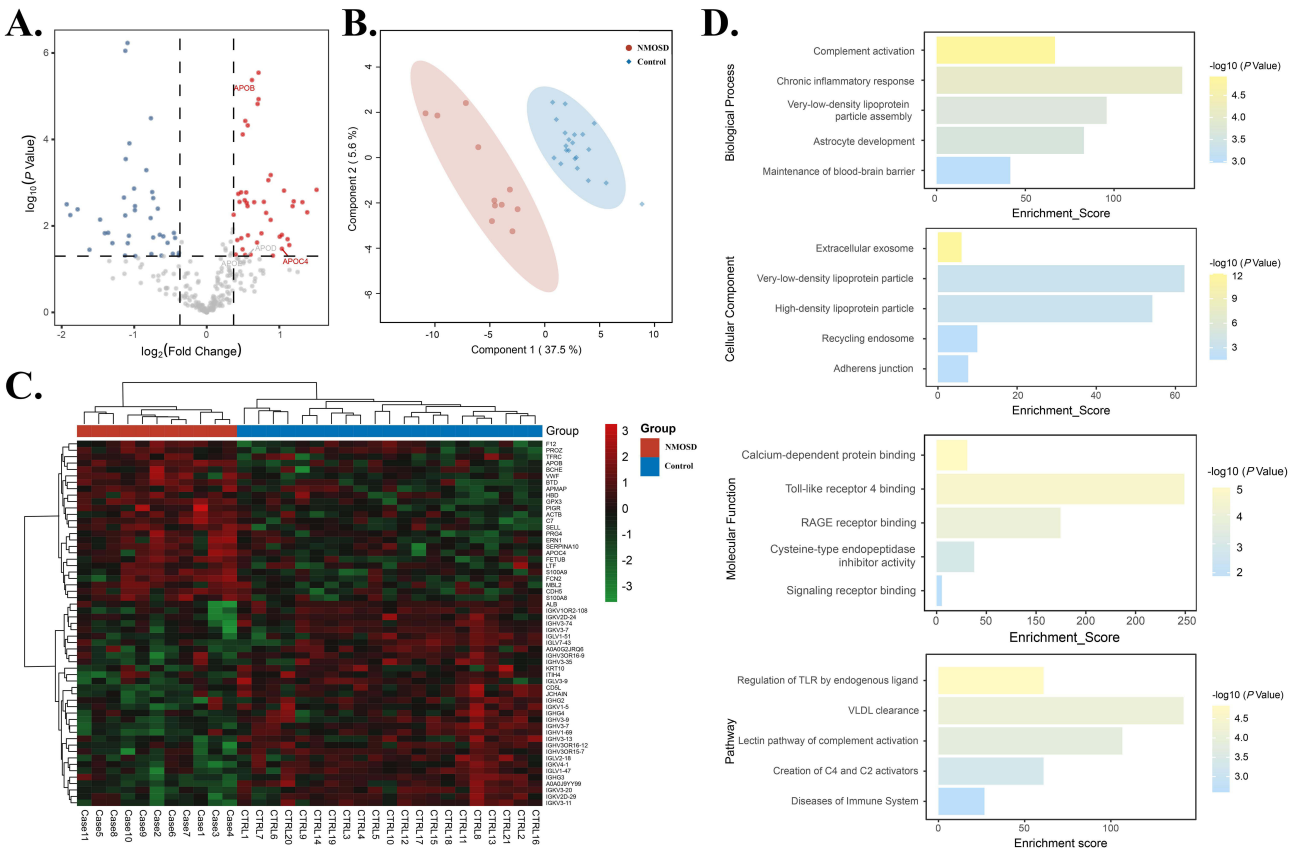

Figure 2 Proteomics analysis of discovery cohort. Proteomic results were visualized through volcano plot (**A**), principal component analysis (PCA) plot (**B**), heatmap (**C**) and pathway enrichment plot (**D**). Volcano plot highlighting differentially expressed proteins with the $p < 0.05$ and the |fold change (FC)| > 1.5.

Diagnostic Accuracy of Serum Lipid Biomarkers in the Validation Cohort

To further assess the discriminatory validity of lipid-related indexes in different types of NMOSD patients, we developed three diagnostic models: one to identify all NMOSD patients (model 1), the second for AQP4-IgG+NMOSD patients (model 2), and the third for double seronegative patients (model 3). The detailed demographic data were listed in Tables 3–5. There were 918 patients enrolled in the validation cohort, with 70% used for the training set and the remaining 30% for the test set. We evaluated the performance of the models using various metrics, including learning curve, confusion matrix, accuracy, recall, positive predictive value (PPV), negative predictive value (NPV) and ROC curve. Figure 3 showed that as the number of samples increased, the training and validation scores curves gradually converged, indicating steady improvement and stable performance of the three models. The confusion matrices revealed that model 1 and model 2 achieved higher accuracy both at 72%, suggesting their superior overall accuracy (Figure 4, Table 6). Model 2 exhibited higher PPV (80%) and recall (0.80) for AQP4-IgG+ NMOSD patients. Notably, model 3 demonstrated an NPV of 77%, indicating its effectiveness in reducing misdiagnosis rate. Furthermore, the results of ROC curve analysis showed that combination of age, TC, TG, ApoA1, ApoB, HDL and LDL in Model 1, 2 and 3 achieved AUC of 0.70, 0.73 and 0.72, respectively (Figure 5). These data highlighted the robustness of lipid biomarkers in diagnostic applications.

Disease Monitoring Performance of Lipid Biomarkers in the NMOSD

We observed that all lipid-related indexes were elevated during the attack stage of NMOSD compared to disease mimics in their attack stages (Figure 6, Table 7). Among NMOSD patients, only serum TC, ApoA1, and HDL were significantly higher during attacks compared to remission ($p < 0.01$, Figure 6, Table 7). Previous studies have indicated a correlation between BMI level and disease progression.[20,21] Thus, we conducted the Cox regression analysis to explore the association between the BMI and the risk of relapse. Results revealed that the overweight BMI was associated with an increased risk of relapse (Figure 7A), the higher BMI was linked to a higher frequency of relapse. To minimize any potential confounding effect caused by overweight, we further examined the longitudinal changes of lipid-related indexes among NMOSD patients with normal BMI. Our findings demonstrated that serum lipid-related indexes (besides TG) decreased as the disease condition stabilized (all $p < 0.05$, Figure 7B).

Predictive Value of Lipid Biomarkers for Relapse

Given these observations, we utilized the longitudinal cohort to further investigate the potential value of the meaningful lipid-related indexes in monitoring NMOSD relapse (vs NMOSD remission, Figure 8). The combination of TC, ApoA1, ApoB, HDL, and LDL exhibited superior diagnostic performance for identifying the attack stage of NMOSD (AUC: 0.861, Sensitivity: 78.9%; Specificity: 89.5%). A combination of lipid biomarkers achieved the AUC of 0.861 in predicting disease relapse, underscoring their utility in proactive clinical management and early intervention strategies.

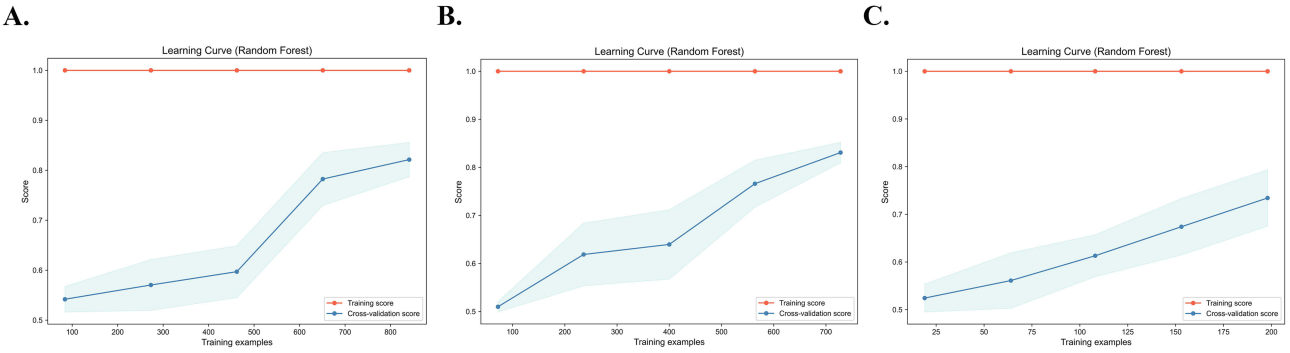

Figure 3 The learning curve of the training score and cross-validation score in diagnostic models. The diagnostic models were for patients in the attack stage with NMOSD (model 1) (**A**), AQP4-IgG+ NMOSD (model 2) (**B**), and double seronegative NMOSD (model 3) (**C**).

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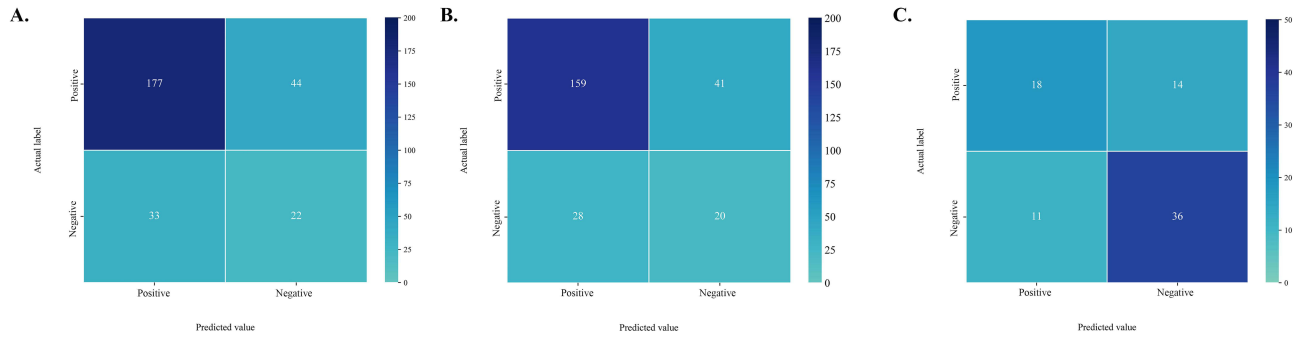

Figure 4 The confusion matrix of the diagnostic models. The diagnostic models were for patients in the attack stage with NMOSD (model 1) (**A**), AQP4-IgG+ NMOSD (model 2) (**B**), and double seronegative NMOSD (model 3) (**C**). The confusion matrix showed the counts of true positive, true negative, false positive, and false negative predictions, which visualized the performance of the models.

Feasibility and Clinical Practice

The serum lipid biomarkers uncovered in this study offer valuable diagnostic insights and are pivotal for the ongoing management of NMOSD. Their straightforward measurement, affordability, and the utilization of established analytical techniques render these biomarkers ideally suited for integration into standard clinical workflows. Consequently, we have harnessed these data to engineer an intuitive application that streamlines the monitoring process, culminating in an economical and efficient sentinel surveillance system. Within this application, we have integrated the formula **(Y= −7.86281+0.7920 * TC-3.88683 * ApoA1+11.59402 * ApoB+5.94912 * HDL-3.35710*LDL)**, enabling the system to predict the risk of NMOSD exacerbations. The Y value represents the score obtained from the established combined diagnostic model. This computed score is used as a diagnostic indicator for ROC analysis, thereby assessing the diagnostic efficacy of the combined indicators. By setting a threshold value above 0.4996, the application facilitates a straightforward "input-index, output-answer" screening process, enhancing both clinical decision-making and patient care.

Discussion

NMOSD is a spectrum of neuroinflammatory disorders and patients often experience frequent relapse and progressive decline in functional abilities.[1] Misdiagnosis and missed diagnosis can have detrimental effects on disease progression

Table 6 The Detail Data of Confusion Matrix

	Model 1	Model 2	Model 3
	NMOSD Attack vs Disease Mimics Attack	AQP4-IgG+NMOSD Attack vs Disease Mimics Attack	Double seronegative NMOSD Attack vs Disease Mimics Attack
Training cohort, n	578	642	184
Test cohort, n	248	276	79
F1 score	0.72	0.72	0.68
True Positive (TP)	159	177	18
False Positive (FP)	28	33	11
True Negative (TN)	20	22	36
False Negative (FN)	41	44	14
Positive Predictive Value (PPV)	80%	80%	56%
Negative Predictive Value (NPV)	42%	40%	77%
Accuracy	72%	72%	68%
Recall	0.80	0.80	0.56

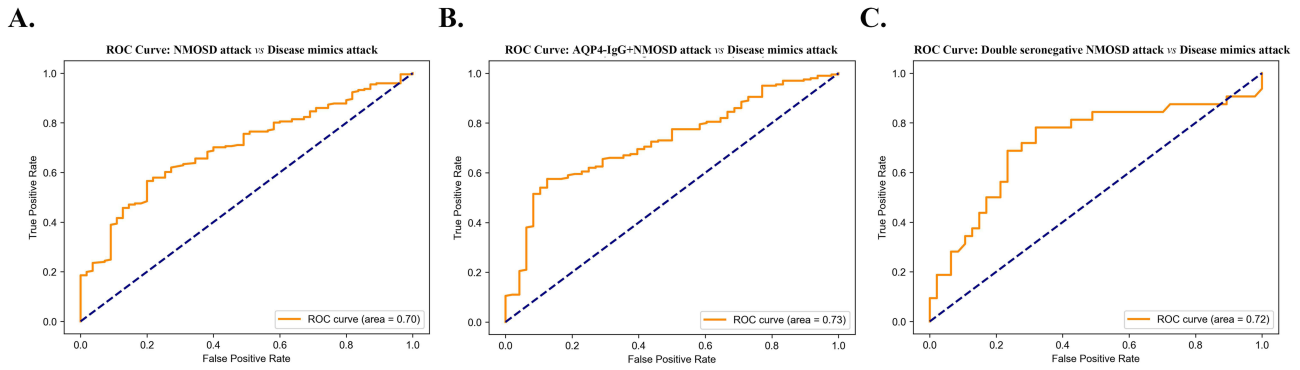

Figure 5 The ROC curves of the three models. The diagnostic models were for patients in the attack stage with NMOSD (model 1) (**A**), AQP4-IgG+ NMOSD (model 2) (**B**), and double seronegative NMOSD (model 3) (**C**).

and prognosis.[22–24] It is imperative to identify potential NMOSD biomarkers for disease diagnosis and monitoring. Therefore, to explore and evaluate potential biomarkers for NMOSD, this study established a discovery cohort and validation cohorts. In the discovery cohort, proteomics results showed that serum apolipoproteins were elevated in NMOSD patients, and enrichment analysis indicated that DEPs were associated with inflammatory responses and lipid regulation. Subsequently, in the cross-sectional validation cohort, we further evaluated the diagnostic accuracy of lipid-related indicators for all NMOSD attack patients, AQP4-IgG+ NMOSD attack patients, and double seronegative attack patients using machine learning, with results showing accuracies of 72%, 72%, and 68%, respectively. Interestingly, the study also indicated that serum lipid levels were significantly elevated in NMOSD patients during the relapse phase and decreased when in the remission. Based on the above results, in the longitudinal cohort, we then developed another model to monitor disease activity using combined lipid-related indicators, with the AUC of 0.861.

We established two different cohorts to decipher the proteomics profiling of NMOSD and analyzed the DEPs in NMOSD and their potential implications. As expected, the DEPs revealed significant enrichment of pathways associated with immune response, astrocyte development, maintenance of the blood-brain barrier (BBB), and lipoprotein metabolism. These findings are highly relevant to the pathological mechanisms underlying NMOSD. In NMOSD, the entry of AQP4 antibodies into central nervous system (CNS) through BBB triggers an immune cascade, resulting in inflammation and subsequent damage to astrocytes, neurons, and myelin.[25–28] The enrichment of immune response pathways supported the involvement of immune dysregulation in NMOSD pathogenesis. Additionally, the findings related to astrocyte development and BBB maintenance highlight the impact of these processes on the integrity of the CNS and its susceptibility to damage in NMOSD. Interestingly, lipoprotein metabolism pathway enrichment suggests the potential involvement of lipids and lipoproteins in NMOSD, which may indicate that lipids have been implicated in neuroinflammatory processes and could play a role in modulating the immune response observed in NMOSD.

Our proteomics results indicated that the lipid profile in NMOSD patients undergoes significant changes (ApoB and ApoC4, $p < 0.05$). Enrichment analysis suggested that the differentially expressed proteins are involved in inflammatory responses and lipid regulation. Then, we demonstrated that lipid-related indicators are elevated during NMOSD relapse, and paired longitudinal data also revealed that lipid levels showed a correlation with disease activity, with levels changing from relapse to remission. Hence, these results suggest that lipid metabolism dysregulation may be involved in the relapse of NMOSD patients. Jiang S et al[29] found that there was an increase in ApoE-rich astrocyte-derived extracellular vesicles in NMOSD patients, and that intracerebral injection of ApoE could reduce the excessive activation of microglia, neuroinflammation, and brain damage in NMOSD mice. Another study[16] indicated that serum low-density lipoprotein (LDL) was positively correlated with neuroinjury biomarkers in NMOSD patients, with LDL penetrating the CNS through a compromised BBB, directly activating microglia. This activation can lead to excessive phagocytosis of myelin debris, inhibit lipid metabolism, increase glycolysis, and ultimately exacerbate myelin damage, highlighting the potential therapeutic value of reducing circulating lipids to alleviate acute demyelination in NMOSD. Consistent with our findings, Jie D et al[30] also reported the correlation between serum LDL and NMOSD patients' relapse phase, indicating

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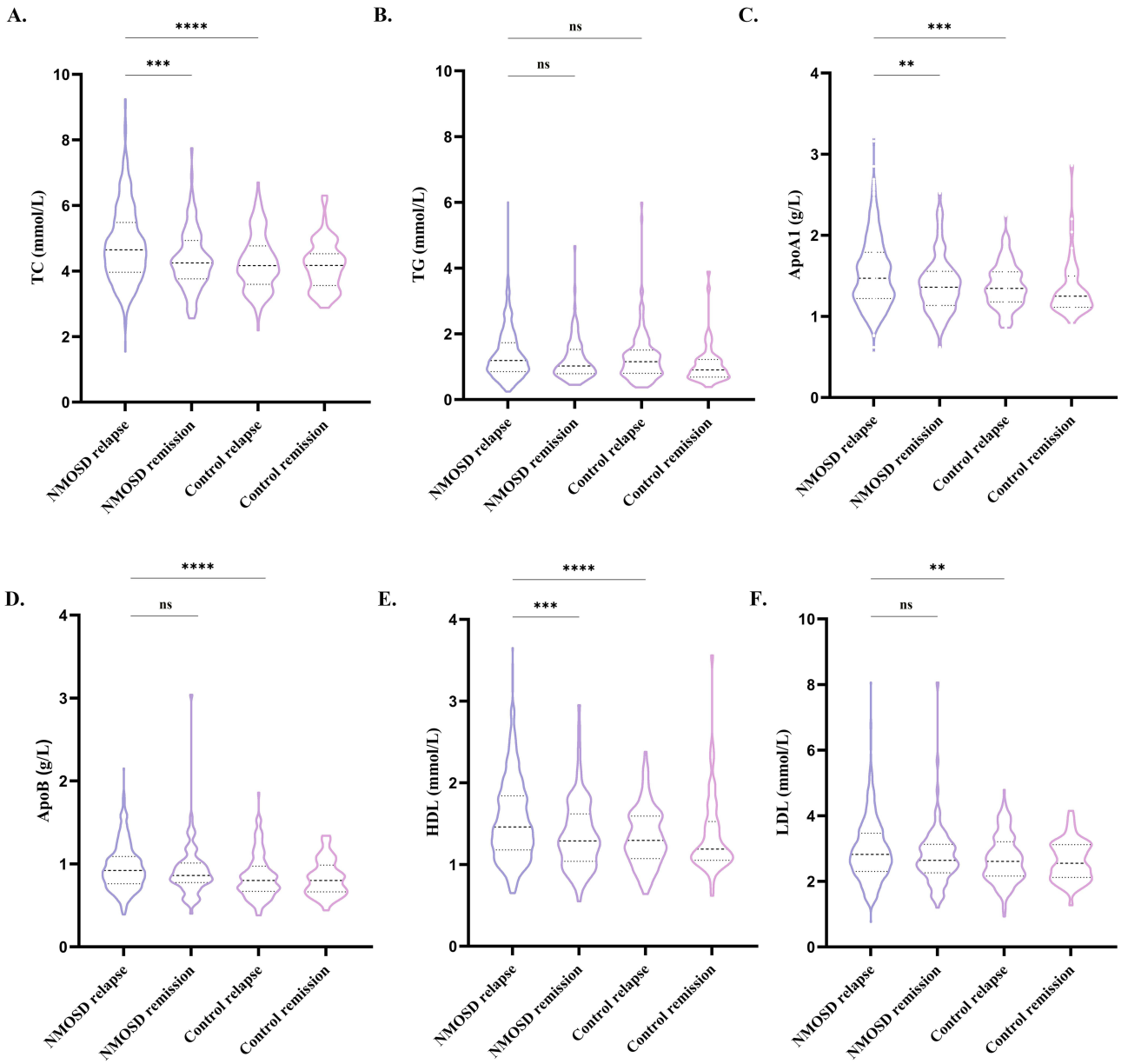

Figure 6 Comparisons of serum TC (**A**), TG (**B**), ApoA1 (**C**), ApoB (**D**), HDL (**E**), and LDL (**F**) levels in the relapse and remission stage of NMOSD and control groups. All patients were in the attack stage. We observed difference of serum lipid-relate indexes levels among NMOSD attack, NMOSD remission, Control attack and Control remission group. Control group included MOGAD, MS, and other nervous system disease (optic neuritis, myelitis,CNS inflammatory demyelinating disease and ALS). Violin plots display medians and interquartile ranges (IQR). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p<0.0001$.

that controlled serum LDL levels can effectively reduce disease recurrence. Collectively, we speculate that alterations in lipid levels may be associated with disease activity, inflammation and neural damage. Therefore, they have become the focus of our subsequent investigations.

Then, we conducted the comprehensive analysis in a large cohort to develop diagnostic models aimed at assessing the discriminatory potential of lipid-related indexes in identifying NMOSD patients during the attack stage. The results demonstrated that the diagnostic models incorporating age, TC, TG, ApoB, LDL, ApoA1 and HDL significantly enhanced the accuracy of diagnosing NMOSD patients in the attack stage, especially for those with AQP4-IgG+ NMOSD. Additionally, this helped reduce the misdiagnosis rate for double seronegative NMOSD patients. Furthermore, by analyzing these indicators across different stages, we delved into their potential for monitoring disease progression in NMOSD patients. Of course, we notice that there are emerging novel biomarkers like serum GFAP and NfL, which have been showed to be significantly

Table 7 Levels of Serum Lipid-Related Indexes in Different Groups

	NMOSD (n = 860)		Disease Mimics (n = 233)		*p* value	
	Attack (n = 747)	Remission (n = 113)	Attack (n = 171)	Remission (n = 62)	NMOSD attack vs NMOSD remission	NMOSD attack vs disease mimics attack
TC (mmol/L)	4.64 (3.96, 5.48)	4.25 (3.76, 4.94)	4.17 (3.60, 4.79)	4.17 (3.56, 4.53)	0.0002	< 0.0001
TG (mmol/L)	1.19 (0.85.1.72)	1.02 (0.79, 1.53)	1.16 (0.80.1.51)	0.91 (0.69, 1.22)	0.0590	0.3672
ApoA1 (g/L)	1.47 (1.22, 1.79)	1.36 (1.14, 1.56)	1.35 (1.18, 1.55)	1.25 (1.11, 1.50)	0.0070	0.0004
ApoB (g/L)	0.92 (0.76, 1.09)	0.86 (0.78, 1.01)	0.80 (0.67, 0.97)	0.80 (0.66, 0.99)	0.2016	< 0.0001
HDL (mmol/L)	1.46 (1.18, 1.84)	1.29 (1.04, 1.62)	1.31 (1.08, 1.60)	1.19 (1.05, 1.53)	0.0005	< 0.0001
LDL (mmol/L)	2.82 (2.30, 3.45)	2.64 (2.26, 3.13)	2.62 (2.16, 3.22)	2.55 (2.12, 3.12)	0.1014	0.0032

Notes: The NMOSD group included AQP4-IgG+NMOSD and double seronegative NMOSD, and disease mimics included MOGAD, MS, other neurological disease (including optic neuritis, myelitis, and ALS).

Abbreviations: NMOSD, neuromyelitis optica spectrum disorder; AQP4, aquaporin-4; AQP4-IgG+NMOSD, AQP4 antibody positive NMOSD; MOG, myelin oligodendrocyte glycoprotein; MOGAD, MOG-antibody-associated diseases; MS, multiple sclerosis; ALS, amyotrophic lateral sclerosis; BMI, body mass index; IQR, inter quartile range; n, number; Variables with 2-tailed $p < 0.05$ were considered significant. Values were displayed as the median and interquartile range (IQR).

elevated during NMOSD relapse and are associated with disease progression,[31,32] indicating that they can also serve as biomarkers for differential diagnosis and monitoring disease activity. However, due to the high demand for ultrasensitive instruments and high costs of serum low abundance biomarkers GFAP and NfL, lipid-related indicators, with their easy-to-test and relatively low-cost, are far more amenable to clinical practice.

Serum levels of ApoA1, HDL, ApoB, and LDL were elevated in patients with NMOSD during the relapse stage. We also found that ApoA1 and HDL were higher during NMOSD attacks compared to the remission stage, while there was a slight increase in ApoB and LDL levels without statistical significance. This suggested that lipid profiles may be associated with the occurrence and severity of NMOSD attacks. Furthermore, our findings revealed that higher BMI was linked to an increased risk of relapse. To mitigate the impact of obesity on the levels of lipid-related indexes, participants with normal BMI were selected to conduct a before-after analysis. Interestingly, a gradual decrease in lipid-related variables (except triglycerides) was observed as the symptoms improved. Through logistic regression and ROC curve analysis on longitudinal data, the findings

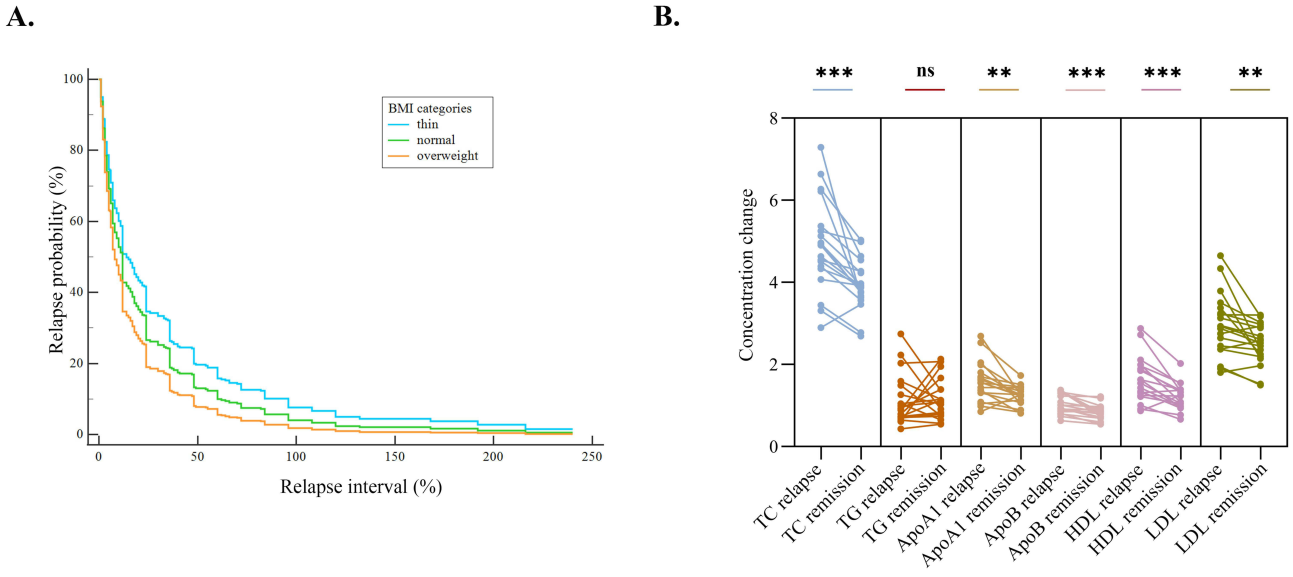

Figure 7 Relationship between the BMI and the relapse risk of NMOSD (**A**), and changes of serum lipid-related indexes in each NMOSD patient (with normal BMI) from attack and remission (**B**). In this part, only AQP4-IgG+NMOSD patients were included. $^{**}p < 0.01$, $^{***}p < 0.001$.

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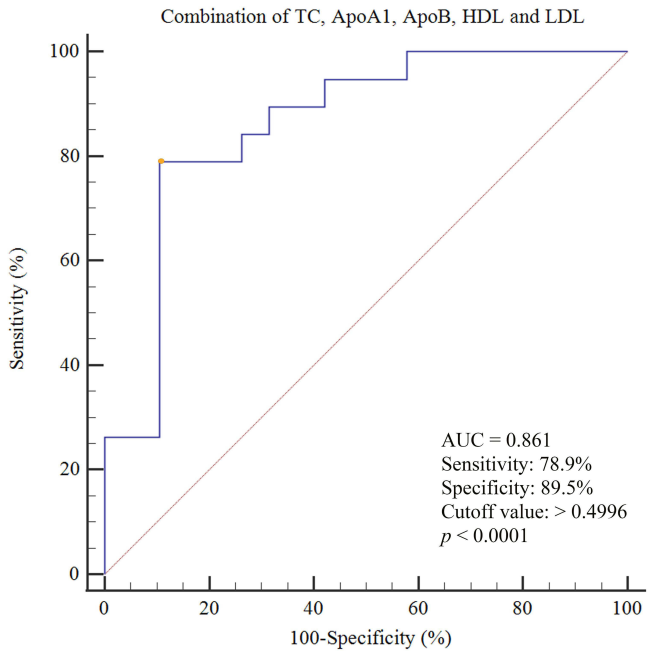

Figure 8 The ROC curve of the diagnostic model to distinguish the attack-stage of NMOSD from the remission-stage of NMOSD. Based on logistic regression analysis, the combining of serum TC, ApoA1, ApoB, HDL, and LDL were used to construct diagnostic model on longitudinal cohort, and the ROC curve analysis was used to evaluate the performance of the model for prediction of the attacks occurrence in NMOSD patients. Evaluation metrics include area under the curve (AUC), Sensitivity and Specificity.

demonstrated that a combination of TC, ApoB, LDL, ApoA1, and HDL could significantly predict the occurrence of attacks in NMOSD patients, which would be a potential signal for the relapse risk in NMOSD patients.

HDL is primarily produced by astrocytes and microglia in the CNS,[33] which possesses anti-inflammatory properties and can suppress immune cell activation and the release of pro-inflammatory molecules. Fellows et al[34] found that higher serum HDL and ApoA1 levels were associated with reduced BBB damage and a decrease of CD80+ and CD80+CD19+ cell in MS patients. ApoA1, a major component of HDL, is considered as the key in anti-inflammatory properties, highlighting its potential involvement in the BBB protection.[35] HDL may have an anti-inflammatory role following the initial demyelinating event, thus supporting the enrichment of DEPs related to BBB maintenance observed in this study. Moreover, ApoA1 and HDL can be directly involved in intracerebral cholesterol transport, thereby affecting CNS function.[36,37] Before-after analysis also indicated that ApoA1 and HDL may continue to exert positive protective effects during attack.

LDL has been implicated in promoting immune cell activation and the production of pro-inflammatory molecules. Additionally, it is vulnerable to oxidation, leading to the generation of ROS and subsequent damage to cells and tissues. Chen M et al[16] revealed that elevated LDL may result in the over-activation of microglia, promoting pro-inflammatory effects and causing the accumulation of myelin debris and lipid droplets. Excessive ApoB may lead to extensive neuronal damage and apoptosis through activation of apoptotic signaling pathways.[38] Researchers have also found significant positive correlation of LDL and disease duration.[39,40] Similarly, NMOSD patients have elevated LDL and TG levels, which are associated with disease activity and progression.[16] The disrupted lipid homeostasis of the CNS further exacerbates demyelination reactions in NMOSD patients. This can be supported by before-after analysis which has demonstrated that as the disease improves, there is a decline in TC, LDL, and ApoB. Interestingly, while HDL, ApoA, LDL, and ApoB may be elevated during the attack stage, patients with NMOSD have a shorter disease duration compared to patients with MS. This may suggest that HDL's protective effect in NMOSD outweighs the damage caused by LDL. Lowering TC, ApoB, and LDL while increasing ApoA1 and HDL may be a beneficial strategy for treating NMOSD.

Taken together, our study provides insights into the serum proteomics profiling of NMOSD and has shed light on the diagnostic and monitoring potential of lipid-related indexes for NMOSD attack. The diagnostic models constructed based on longitudinal and cross-sectional cohorts not only improve the diagnosis, reduce misdiagnosis risk of patients with double seronegative NMOSD and further support the utility of lipid-related indexes in NMOSD management. Although

our study positively explores the novel application of lipids in clinic, it is important to note that further research is required to fully elucidate the underlying mechanisms involved in this process. In addition, another limitation of this study is that it did not analyze the effects of factors such as regional differences, gender distribution, age group and disease severity on the generalizability of these findings, which needs to be explored in a broader research population in the future. Meanwhile, we need to supplement external validation and cross-sectional studies to further refine these results.

Data Sharing Statement

Data will be made available on request from the correspondence author.

Ethics Approval

This research involved human samples and complied with the Declaration of Helsinki. It was approved by Ethics Committee Of Chinese PLA General Hospital (No. S2021-128-01, No. S2021-128-02) and informed consent was obtained from participants.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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