

Elucidating Biological Links in Autism Spectrum Disorder: From Blood-Based Risk Factor Identification to a Validated Nomogram for Early Risk Stratification

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Objective: Blood-based biomarkers are valuable for investigating Autism Spectrum Disorder (ASD). This study aimed to identify key blood-based risk factors for ASD and to develop and validate a clinical model for predicting disease risk.

Methods: We retrospectively analyzed data from 879 children, including patients with ASD and healthy controls. After using propensity score matching to balance for age and sex, we employed multivariate logistic regression to identify independent risk factors from routine blood parameters, vitamins, and trace elements. We then characterized dose-response relationships and a nomogram for ASD risk prediction was constructed and internally validated.

Results: Four independent risk factors were identified: calcium, serum iron, vitamin D, and platelet distribution width (PDW). Lower levels of serum iron and vitamin D, along with higher PDW levels, were significantly associated with an increased risk of ASD. Calcium exhibited a non-linear relationship, with risk peaking at a concentration of 1.13 mmol/L. The nomogram based on these four markers demonstrated strong predictive performance, with sensitivities of 84.6% and 78.6% and specificities of 73.4% and 79.0% for the training and validation sets, respectively.

Conclusion: These findings highlight the feasibility of calcium, iron, vitamin D, and PDW as independent blood-based risk factors for ASD predicting and elucidated their specific patterns of association with ASD prevalence. The nomogram ASD risk prediction model, constructed utilizing these key markers, demonstrated commendable discrimination, calibration, and clinical utility. This model holds considerable promise as an efficacious tool for early ASD screening and risk assessment, with significant potential for clinical translation.

Keywords: autism spectrum disorder, ASD, propensity score matching, PSM, clinical risk factors, dose-response relationship, restricted cubic spline, RCS, nomogram prediction model

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by persistent impairments in social communication and the presence of restricted, repetitive behaviors. Accumulating evidence suggests its etiology is not solely genetic but involves a complex interplay between genetic predispositions and environmental factors, with gene-environment interactions and epigenetic modifications recognized as pivotal influences.¹⁻⁴

Blood-based biomarkers offer a clinically accessible window into the disorder's biological perturbations, particularly for assessing nutritional deficiencies and metabolic disturbances modulated by environmental influences.^{5,6} Prior research

has consistently demonstrated distinct differences in blood marker profiles, showing that children with ASD frequently exhibit nutritional deficiencies and metabolic dysregulation.^{7–9} For instance, studies have reported significantly lower levels of trace elements like zinc and magnesium,¹⁰ and vitamin D insufficiency has also been implicated in an elevated risk of ASD.¹¹ Despite these advancements,^{12,13} the clinical translation of these findings remains challenging. Prior investigations are often constrained by limited sample sizes, which compromise generalizability,¹⁴ and a narrow focus on individual markers. This fragmented approach overlooks the complex interactive networks among multiple indicators and has hindered the development of comprehensive models that reflect the multifaceted pathology of ASD.¹⁵

To address this critical gap, the primary objective of the present study was to comprehensively explore the specific and synergistic associations among a panel of trace elements, vitamins, and routine blood parameters with the risk of ASD. We employed a rigorous multivariate approach, including Propensity Score Matching (PSM) and bidirectional stepwise regression, to identify the most potent and independently significant peripheral blood-based risk factors. Furthermore, the study utilizes Restricted Cubic Spline (RCS) models to meticulously characterize the precise non-linear dose-response relationships between these pivotal indicators and ASD risk, thereby elucidating their potential underlying mechanisms. Ultimately, based on these independently identified factors, a practical, validated nomogram for early ASD risk prediction was developed and rigorously validated to facilitate efficacious risk stratification, early screening, and prompt intervention in general clinical settings.

Materials and Methods

Subjects

This retrospective study collected clinical data from 879 children who presented to the Dalian Women and Children's Centre (Group) between January 2022 and October 2023. Participants were stratified into an ASD group ($n = 338$) or a healthy control group ($n = 541$) based on their diagnostic status. The ASD cohort comprised children aged 1 to 15 years (average age was 4.38 ± 2.40), who rigorously met the diagnostic criteria for ASD as defined by the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), and who had no history of prior interventional treatment for ASD. The healthy control group consisted of typically developing children within the same age range (average age was 4.58 ± 2.32). Exclusion criteria for both groups encompassed a history of head trauma, diagnosis of other neurological disorders, presence of severe somatic diseases, uncontrolled epileptic seizures, or incomplete clinical data records. The study strictly adhered to ethical guidelines for medical research; all diagnostic evaluations and blood tests were performed with explicit informed consent obtained from the patients or their legal guardians. All collected data were subsequently anonymized and maintained with strict confidentiality. Finally, all enrolled participants underwent a further screening process via Propensity Score Matching (PSM) for definitive inclusion in the study.

Blood Indicators

All blood sample data were retrieved from the comprehensive electronic medical record system of the Dalian Women and Children's Centre (Group). This information included patient sex, age, and a complete panel of peripheral blood test results. A total of 28 distinct blood markers were meticulously collected, comprising 15 routine hematological parameters, 8 trace elements, and 5 vitamin levels. A detailed list of all markers and their reference ranges is provided in the [Supplementary Methods](#).

Statistical Analysis

The normality of continuous data was assessed using the Kolmogorov–Smirnov test. Data are presented as mean $\pm$ standard deviation or median (interquartile range) and were compared using independent sample *t*-tests or Wilcoxon rank-sum tests, respectively. Categorical data are presented as frequencies (%) and were compared using the Pearson χ^2 test. For the significance *p*-value, a multiple comparison correction method (Benjamini–Hochberg, FDR) was used to control false positives.¹⁶

To control for the confounding effects of age and sex, we performed 1:1 propensity score matching (PSM) to balance the ASD and control groups. Independent risk factors were then identified using a two-stage regression analysis: an initial

screening with univariate logistic regression (FDR-corrected $p < 0.1$), followed by a multivariate logistic regression to determine the final predictors (FDR-corrected $p < 0.05$). Additionally, multicollinearity among independent variables in the multivariate logistic regression model was assessed via the variance inflation factor (VIF) and tolerance. Sensitivity analysis (sequential exclusion of variables with mild multicollinearity and model refitting) was performed to verify the stability of core regression coefficients (i.e., consistency of odds ratios [OR] and statistical significance p -value), thus ensuring robust study. RCS models were used to characterize the dose-response relationships between these key factors and ASD risk. After randomly splitting the matched dataset ($n = 676$) into training (70%) and validation (30%) sets, we developed two nomogram prediction models: a full-variable model (Model 1) and a streamlined model comprising only the core risk factors (Model 2). The models' performance was assessed for discrimination (C-index, ROC curves) and calibration (calibration curves), and internally validated via bootstrap resampling. The DeLong test was used to compare the models' area under the curve (AUC). All analyses were performed in R software (v. 4.4.0), with further details provided in the [Supplementary Methods](#).

Results

Comparison of Information Between Groups Before and After Propensity Score Matching

The application of PSM facilitated a successful 1:1 matching of 338 ASD patients with 338 healthy controls, based on the covariates of age and sex. [Table 1](#) illustrates the comparative characteristics of the two groups before and after the PSM procedure. Prior to PSM, both age and sex exhibited statistically significant differences between the two groups ($p < 0.001$). The SMD was 0.36 for sex and 0.40 for age, indicating substantial baseline imbalances. Following PSM, these disparities were effectively eliminated, with no statistically significant differences observed for either age ($p = 0.233$) or sex ($p = 0.595$). Concomitantly, the SMD for sex was markedly reduced to 0.04, and for age, it decreased to 0.08, both well below the conventional threshold of 0.1 for covariate balance. Despite this successful demographic matching, analyses of data before and after PSM consistently indicated persistent differences between the ASD and healthy groups concerning hematological, nutritional, and heavy metal-related indicators ([Table 1](#)).

To further corroborate the inter-group balance post-PSM, the distributions of age and sex for both the ASD and healthy cohorts were visually represented, as depicted in [Supplementary Figure 1](#). Density plots compellingly demonstrated a substantial increase in the overlap of the age variable between the groups, while bar charts clearly indicated a significant reduction in gender disparity. In summation, the PSM methodology proved highly effective in neutralizing pre-existing differences in age and sex, thereby achieving the critical objective of covariate balance for this investigation.

Screening for ASD-Related Significant Independent Risk Factors Based on Univariate and Multivariate Logistic Regression

[Table 2](#) delineates the ASD risk factors identified through a comprehensive analysis involving both univariate and multivariate logistic regression models. Initial univariate logistic regression analysis revealed statistically significant differences (FDR-corrected $p < 0.1$) in 16 blood markers between the ASD and healthy control groups, suggesting their potential as risk factors for ASD. These markers included: PDW (95% CI: 1.375–1.554, OR = 1.460), MCHC (95% CI: 0.946–0.978, OR = 0.962), Hematocrit (95% CI: 1.010–1.121, OR = 1.063), MCH (95% CI: 1.155–1.478, OR = 1.303), MCV (95% CI: 1.113–1.216, OR = 1.162), RDW-SD (95% CI: 1.031–1.148, OR = 1.087), RDW-CV (95% CI: 0.680–0.997, OR = 0.826), PCT (95% CI: 0.001–0.113, OR = 0.012) from routine blood parameters; Copper (95% CI: 0.858–0.949, OR = 0.903), Iron (95% CI: 0.400–0.554, OR = 0.473), Calcium (95% CI: 0.017–0.054, OR = 0.031), Magnesium (95% CI: 0.004–0.021, OR = 0.009), Zinc (95% CI: 1.033–1.057, OR = 1.045) from trace elements; and Vitamin D (95% CI: 0.938–0.966, OR = 0.953), Vitamin K1 (95% CI: 0.525–0.910, OR = 0.697), and Vitamin K2 (95% CI: 0.107–0.708, OR = 0.294) from the vitamin panel.

In addition, multicollinearity among independent variables in the multivariate logistic regression model was evaluated using the variance inflation factor (VIF) and tolerance. Results showed that the VIF values of all included variables ranged from 1.09 to 5.4 ([Supplementary Table 1](#)), all below the threshold for severe multicollinearity ($VIF \geq 10$). Only

Table I Comparative Analysis of Clinical Characteristics Between ASD and Healthy Populations in Pre- and Post- PSM Phases

Characteristic	Pre-PSM Data				Post-PSM Data			
	Overall	Control Group	ASD Group	p-value ^b	Overall	Control Group	ASD Group	p-value ^b
	N = 879 ^a	N = 541 ^a	N = 338 ^a		N = 676 ^a	N = 338 ^a	N = 338 ^a	
Sex								
Male	559 (64%)	309 (57%)	250 (74%)	<0.001	506 (75%)	256 (76%)	250 (74%)	0.595
Female	320 (36%)	232 (43%)	88 (26%)		170 (25%)	82 (24%)	88 (26%)	
Age	4(3.00, 7.00)	5(3.00, 7.00)	4(3.00, 5.00)	<0.001	4(3.00, 6.00)	4(3.00, 6.00)	4(3.00, 5.00)	0.233
Copper	17.45(15.28, 19.54)	17.77(15.66, 19.87)	17(14.73, 18.80)	<0.001	17.22(15.13, 19.48)	17.67(15.65, 20.00)	17(14.73, 18.80)	<0.001
Iron	7.58(6.76, 8.20)	7.89(7.30, 8.43)	7.03(5.90, 7.82)	<0.001	7.48(6.68, 8.14)	7.87(7.34, 8.41)	7.03(5.90, 7.82)	<0.001
Calcium	1.57(1.10, 1.71)	1.63(1.51, 1.75)	1.13(1.03, 1.55)	<0.001	1.53(1.08, 1.70)	1.63(1.51, 1.75)	1.13(1.03, 1.55)	<0.001
Magnesium	1.48(1.28, 1.61)	1.55(1.43, 1.65)	1.31(1.08, 1.47)	<0.001	1.45(1.24, 1.59)	1.55(1.44, 1.64)	1.31(1.08, 1.47)	<0.001
Zinc	79.87(71.69, 91.03)	78.36(68.69, 86.90)	82.79(77.33, 98.12)	<0.001	79.76(72.18, 91.75)	77.88(68.05, 85.43)	82.79(77.33, 98.12)	<0.001
Manganese	0.48(0.36, 0.64)	0.47(0.36, 0.63)	0.49(0.35, 0.66)	0.66	0.48(0.36, 0.64)	0.47(0.36, 0.63)	0.49(0.35, 0.66)	0.352
Selenium	98.56(84.48, 109.65)	98.65(84.48, 109.98)	97.79(85.51, 109.62)	0.985	98.67(85.92, 109.73)	99.72(86.20, 110.05)	97.79(85.51, 109.62)	0.483
Lithium	0.84(0.63, 1.19)	0.84(0.58, 1.19)	0.86(0.67, 1.19)	0.129	0.86(0.64, 1.19)	0.85(0.56, 1.21)	0.86(0.67, 1.19)	0.402
Vitamin A	0.37(0.33, 0.43)	0.38(0.33, 0.44)	0.37(0.32, 0.42)	0.008	0.37(0.33, 0.43)	0.38(0.33, 0.44)	0.37(0.32, 0.42)	0.042
Vitamin E	9.4(8.00, 10.90)	9.2(7.90, 10.70)	9.5(8.00, 11.40)	0.232	9.4(8.00, 11.10)	9.4(8.00, 10.80)	9.5(8.00, 11.40)	0.584
Vitamin D	30.62(24.85, 38.88)	32.42(26.04, 42.20)	28.48(23.11, 35.45)	<0.001	30.6(24.71, 38.53)	33.04(26.73, 42.42)	28.48(23.11, 35.45)	<0.001
Vitamin K1	0.55(0.36, 0.78)	0.55(0.37, 0.81)	0.55(0.35, 0.77)	0.223	0.56(0.36, 0.83)	0.59(0.37, 0.89)	0.55(0.35, 0.77)	0.03
Vitamin K2	0.12(0.07, 0.20)	0.13(0.08, 0.23)	0.11(0.07, 0.17)	0.001	0.12(0.07, 0.20)	0.13(0.07, 0.24)	0.11(0.07, 0.17)	0.009
White blood count	7.21(6.03, 8.47)	7.19(6.05, 8.50)	7.3(6.02, 8.46)	0.988	7.23(6.06, 8.44)	7.2(6.09, 8.40)	7.3(6.02, 8.46)	0.962
Red cell count	4.53(4.32, 4.76)	4.55(4.34, 4.78)	4.48(4.29, 4.71)	0.013	4.52(4.31, 4.74)	4.54(4.34, 4.78)	4.48(4.29, 4.71)	0.049
Platelet count	308(265.00, 362.00)	312(266.00, 364.00)	305(263.00, 357.00)	0.263	310(265.00, 362.00)	314.5(266.00, 365.00)	305(263.00, 357.00)	0.167
PDW	14(9.80, 15.70)	10.5(9.30, 15.40)	15.6(15.30, 15.90)	<0.001	15.3(9.90, 15.70)	10.4(9.21, 15.48)	15.6(15.30, 15.90)	<0.001
Hemoglobin	126(121.00, 132.00)	126(120.00, 132.00)	126(121.00, 131.00)	0.819	126(121.00, 131.00)	126(120.25, 131.00)	126(121.00, 131.00)	0.642
MCHC	338(332.00, 345.00)	340(333.00, 346.00)	337(331.00, 343.00)	<0.001	338(332.00, 344.00)	340(333.00, 347.00)	337(331.00, 343.00)	<0.001
Neutrophil	2.52(1.89, 3.39)	2.48(1.85, 3.37)	2.68(1.96, 3.41)	0.11	2.53(1.88, 3.36)	2.42(1.82, 3.32)	2.68(1.96, 3.41)	0.05
Lymphocyte	3.68(2.93, 4.78)	3.75(2.96, 4.81)	3.62(2.89, 4.77)	0.641	3.69(3.02, 4.81)	3.78(3.05, 4.91)	3.62(2.89, 4.77)	0.283
Monocyte	0.4(0.33, 0.48)	0.4(0.32, 0.49)	0.4(0.34, 0.47)	0.828	0.4(0.33, 0.48)	0.4(0.32, 0.49)	0.4(0.34, 0.47)	0.647
Hematocrit	37.2(35.40, 39.30)	37.2(35.30, 39.20)	37.2(35.50, 39.48)	0.244	37.05(35.40, 39.20)	36.9(35.20, 38.80)	37.2(35.50, 39.48)	0.052
MCH	28(27.10, 28.80)	27.9(27.00, 28.70)	28.1(27.40, 28.90)	0.002	28(27.20, 28.70)	27.85(27.00, 28.50)	28.1(27.40, 28.90)	<0.001
MCV	82.6(79.90, 84.75)	81.9(79.30, 84.30)	83.5(81.43, 85.40)	<0.001	82.5(79.90, 84.70)	81.6(79.10, 83.70)	83.5(81.43, 85.40)	<0.001
RDW_SD	37.9(36.10, 39.80)	37.6(35.80, 39.40)	38.5(37.10, 40.30)	<0.001	38.1(36.20, 40.10)	37.6(35.80, 39.88)	38.5(37.10, 40.30)	<0.001
RDW_CV	12.6(12.20, 13.10)	12.7(12.30, 13.10)	12.6(12.20, 13.10)	0.082	12.6(12.20, 13.20)	12.7(12.30, 13.20)	12.6(12.20, 13.10)	0.016
PCT	0.28(0.24, 0.32)	0.28(0.24, 0.33)	0.26(0.23, 0.31)	<0.001	0.27(0.23, 0.32)	0.28(0.24, 0.33)	0.26(0.23, 0.31)	<0.001

Notes: ^an (%); Median (IQR). ^bPearson's Chi-squared test; Wilcoxon rank sum test; p-value with FDR correction.

Abbreviations: ASD, autism spectrum disorder; PSM, propensity score matching; PDW, platelet distribution width; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; RDW-SD, red cell distribution width - standard deviation; RDW-CV, red cell distribution width - coefficient of variation; PCT, plateletcrit.

Table 2 Factors Influencing ASD: Univariate and Multivariate Stepwise Logistic Regression Analyses

Variable	Univariable Regression				Multivariable Regression			
	p-value ^a	OR	95% CI		p-value ^a	OR	95% CI	
			Lower	Upper			Lower	Upper
Copper	<0.001*	0.903	0.858	0.949	0.622	1.022	0.957	1.092
Iron	<0.001*	0.473	0.400	0.554	0.021**	0.740	0.601	0.910
Calcium	<0.001*	0.031	0.017	0.054	0.021**	0.286	0.119	0.686
Magnesium	<0.001*	0.009	0.004	0.021	0.211	0.346	0.098	1.221
Zinc	<0.001*	1.045	1.033	1.057	0.211	1.012	0.998	1.027
Manganese	0.367	1.377	0.794	2.401				
Selenium	0.778	0.999	0.990	1.007				
Lithium	0.645	1.049	0.876	1.259				
Vitamin A	0.173	0.215	0.032	1.395				
Vitamin E	0.645	1.016	0.952	1.085				
Vitamin D	<0.001*	0.953	0.938	0.966	0.021**	0.973	0.955	0.991
Vitamin K1	0.022*	0.697	0.525	0.910	0.884	1.036	0.712	1.507
Vitamin K2	0.023*	0.294	0.107	0.708	0.060	0.271	0.092	0.797
White blood count	0.634	0.977	0.906	1.054				
Red cell count	0.131	0.674	0.433	1.042				
Platelet count	0.125	0.998	0.996	1.000				
PDW	<0.001*	1.460	1.375	1.554	<0.001**	1.290	1.180	1.410
Hemoglobin	0.376	1.009	0.992	1.027				
MCHC	<0.001*	0.962	0.946	0.978	0.427	1.018	0.986	1.051
Neutrophil	0.367	1.064	0.954	1.188				
Lymphocyte	0.478	0.954	0.856	1.062				
Monocyte	0.367	0.535	0.180	1.567				
Hematocrit	0.042*	1.063	1.010	1.121	0.249	0.945	0.878	1.017
MCH	<0.001*	1.303	1.155	1.478	0.884	1.025	0.739	1.421
MCV	<0.001*	1.162	1.113	1.216	0.400	1.066	0.959	1.185
RDW_SD	0.006*	1.087	1.031	1.148	0.211	0.911	0.822	1.011
RDW_CV	0.094*	0.826	0.680	0.997	0.499	1.168	0.825	1.655
PCT	<0.001*	0.012	0.001	0.113	0.713	2.111	0.102	43.700

Notes: ^aPearson's Chi-squared test; Wilcoxon rank sum test. *p-value < 0.1 (Univariate, FDR correction), **p-value < 0.05 (Multivariate, FDR correction).

Abbreviations: OR, odds ratio; CI, confidence interval.

MCH (VIF = 5.4, tolerance = 0.185) and MCV (VIF = 5.1, tolerance = 0.196) exhibited mild multicollinearity, while the remaining variables had VIF values < 5, indicating no significant collinearity interference. Sensitivity analysis via sequential exclusion of MCH or MCV ([Supplementary Table 1](#)) confirmed that the odds ratios (OR) and statistical significance of core independent variables remained essentially unchanged. Furthermore, MCH and MCV themselves were not statistically significant in the multivariate logistic regression model (FDR-corrected $p = 0.884$ and 0.400 , respectively). Collectively, only mild multicollinearity existed in the model, which did not materially interfere with the core conclusions, thus verifying the robustness of the multivariate logistic regression results. Further rigorous analysis, integrating these initially identified indicators as independent variables, was conducted via multivariate logistic regression. This statistical procedure ultimately pinpointed four key indicators as significant independent risk factors for ASD (FDR-corrected $p < 0.05$): Iron (95% CI: 0.601–0.910, OR = 0.740, FDR-corrected $p = 0.021$), Calcium (95% CI:

0.119–0.686, OR = 0.286, FDR-corrected $p = 0.021$), Vitamin D (95% CI: 0.955–0.991, OR = 0.973, FDR-corrected $p = 0.021$), and PDW (95% CI: 1.180–1.410, OR = 1.290, FDR-corrected $p < 0.001$). These compelling findings provide robust evidence that calcium, iron, vitamin D, and PDW are significantly associated with the occurrence of ASD, as comprehensively detailed in Table 2.

Predictive Efficacy and Dose-Response Relationship Analysis of Independent ASD Risk Factors

We assessed the individual predictive efficacy of independent ASD risk factors—calcium, iron, vitamin D, and PDW—identified via multivariate Logistic regression, using ROC curves. Figure 1a illustrates these findings: calcium yielded an AUC of 0.765 (95% CI: 0.728–0.802), iron an AUC of 0.714 (95% CI: 0.676–0.753), PDW an AUC of 0.747 (95% CI: 0.709–0.785), and vitamin D an AUC of 0.643 (95% CI: 0.602–0.684). Notably, calcium, iron, and PDW consistently demonstrated AUC values exceeding 0.7, indicating their significant predictive utility for ASD and potential as clinical biomarkers. In contrast, vitamin D's predictive efficacy (AUC = 0.643) was comparatively limited, suggesting it is insufficient as a standalone predictive indicator.

To meticulously elucidate the dose-response relationships between the significant independent risk factors (iron, calcium, vitamin D, and PDW) and the risk of ASD, four distinct RCS models were constructed. In these models, the changes in serum assay values for each marker were plotted on the x-axis, while the corresponding Odds Ratio values for ASD risk were depicted on the y-axis, and the light blue shaded area represents the 95% confidence interval (95% CI). The results revealed unique association patterns for each biomarker with ASD susceptibility: Calcium exhibited a statistically significant non-linear association with ASD risk (Overall $p < 0.001$, Nonlinearity $p < 0.001$), characterized by two discernible inflection points at 1.13 mmol/L and 1.54 mmol/L. As illustrated in Figure 1b, a notable risk reversal was observed at the 1.13 mmol/L inflection point: when serum calcium levels were below 1.13 mmol/L, the risk of ASD incrementally increased with rising calcium concentrations. Conversely, when calcium levels surpassed 1.13 mmol/L, the risk of ASD demonstrated a subsequent decrease with further increases in calcium. Once serum calcium concentrations exceed 1.54 mmol/L, no association is observed between calcium levels and ASD risk. In contrast, PDW demonstrated

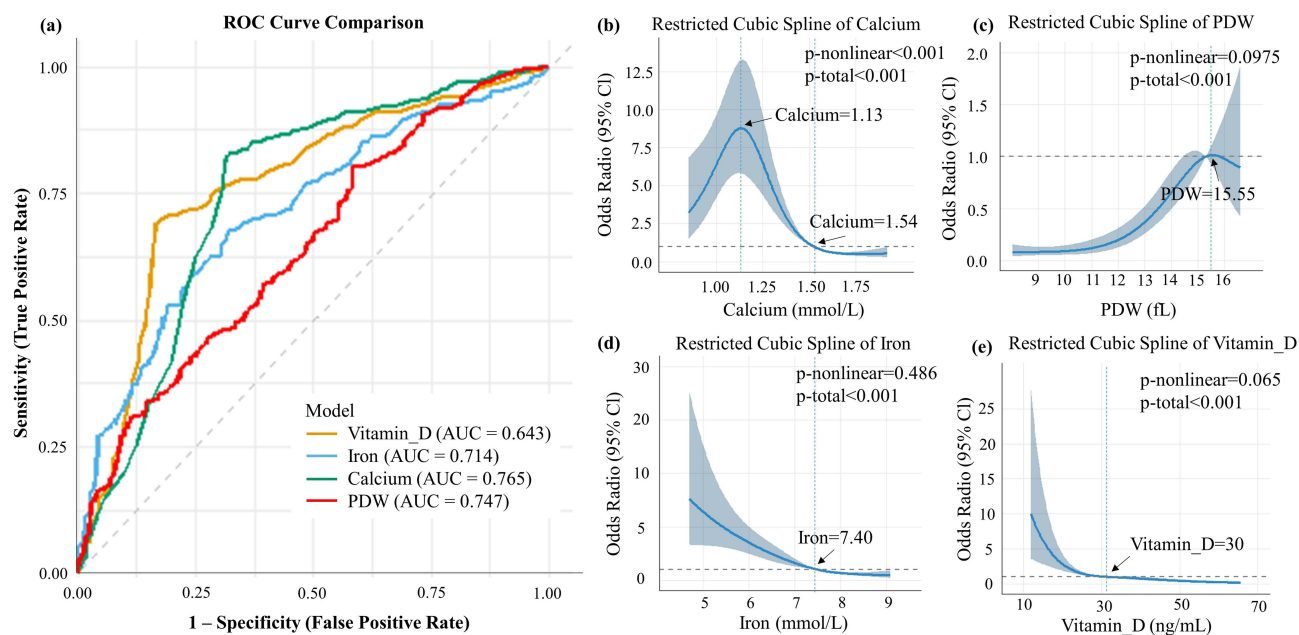


Figure 1 Predictive Efficacy and Dose-Response Relationships of Independent Risk Factors. (a) Receiver Operating Characteristic (ROC) curves show the individual predictive performance of four significant independent risk factors for Autism Spectrum Disorder (ASD). (b) A dose-response curve for calcium, derived from a Restricted Cubic Spline (RCS) model, illustrates a significant non-linear relationship with ASD risk. (c) The dose-response relationship for platelet distribution width (PDW) demonstrates a positive linear correlation with ASD risk. (d) The dose-response curve for iron shows a negative linear correlation with the risk of ASD. (e) The dose-response relationship for vitamin D indicates a negative linear correlation with ASD risk.

a significant positive linear correlation with ASD risk (Overall $p < 0.001$, Nonlinearity $p = 0.0975$). Lower PDW levels were also associated with a lower risk of ASD, and the risk of ASD increased in tandem with rising PDW levels, the risk disease slightly decreased above a PDW level of 15.55 fL, as depicted in [Figure 1c](#). For both Iron and Vitamin D, no significant non-linear associations with ASD risk were observed. Instead, both markers demonstrated a predominantly linear negative association with ASD risk. The risk decreased as concentrations increased, stabilizing once iron levels reached 7.40 mmol/L and vitamin D reached 30 ng/mL. ([Figures 1d and e](#)).

Dataset Partitioning and Nomogram Prediction Model Construction

Training and Validation Set Partitioning

The training set comprised 473 participants, consisting of 245 (51.7%) ASD patients and 228 (48.3%) healthy controls. The validation set included 203 participants, with 100 (49.3%) ASD patients and 103 (50.7%) healthy controls. A comprehensive comparison of baseline characteristics between the training and validation sets revealed no statistically significant differences (FDR-corrected $p = 0.05$), as detailed in [Table 3](#).

Table 3 Comparison of General Patient Information Between Training and Validation Sets

Characteristic	Training Set	Validation Set	p-value ^b
	N = 473 ^a	N = 203 ^a	
Sex			
Male	361(76.4%)	151(74.2%)	0.622
Female	112(23.6%)	52(25.8%)	
Age	4(3.00,6.00)	4(3.00,5.50)	0.67
Copper	17.24(15.06,19.45)	17.2(15.28,19.68)	0.15
Iron	7.47(6.53,8.05)	7.53(6.72,8.34)	
Calcium	1.52(1.08,1.70)	1.55(1.08,1.70)	0.723
Magnesium	1.45(1.23,1.58)	1.45(1.25,1.61)	0.424
Zinc	79.59(71.64,91.03)	79.9(74.20,92.11)	0.515
Manganese	0.48(0.36,0.64)	0.48(0.35,0.63)	0.677
Selenium	97.55(85.51,110.67)	100.63(86.45,107.35)	0.372
Lithium	0.85(0.64,1.19)	0.89(0.64,1.22)	
Vitamin A	0.37(0.33,0.43)	0.36(0.32,0.43)	0.099
Vitamin E	9.4(7.90,10.90)	9.50(8.40,11.40)	0.148
Vitamin D	30.62(24.68,38.51)	30.14(24.80,38.54)	0.888
Vitamin K1	0.55(0.36,0.85)	0.59(0.36,0.81)	0.776
Vitamin K2	0.11(0.07,0.19)	0.12(0.06,0.20)	
White blood count	7.2(6.05,8.41)	7.25(6.12,8.50)	0.404
Red cell count	4.50(4.30,4.73)	4.53(4.32,4.79)	0.773
Platelet count	313(267.00,365.00)	305(259.00,351.50)	0.141
PDW	15.3(9.94,15.70)	15.3(9.90,15.70)	0.695
Hemoglobin	126(121.00,131.00)	126(120.00,131.00)	0.979
MCHC	338.23±9.72	338.31±8.61	0.228
Neutrophil	2.5(1.84,3.35)	2.61(1.96,3.40)	
Lymphocyte	3.77(2.94,4.95)	3.62(3.08,4.48)	0.385
Monocyte	0.4(0.33,0.47)	0.41(0.33,0.50)	0.471
Hematocrit	37(35.40,39.10)	37.3(35.60,39.30)	0.343
MCH	28(27.10,28.80)	27.9(27.25,28.50)	0.67
MCV	82.5(79.80,84.70)	82.7(80.35,84.55)	0.682
RDW_SD	38.1(36.20,40.10)	38.2(36.20,40.15)	
RDW_CV	12.6(12.20,13.20)	12.7(12.30,13.15)	0.279

(Continued)

Table 3 (Continued).

Characteristic	Training Set	Validation Set	<i>p</i> -value ^b
	N = 473 ^a	N = 203 ^a	
PCT	0.27(0.23,0.33)	0.26(0.23,0.31)	0.154
Group (ASD/Control)	51.7%/48.3%	49.3%/50.7%	0.615

Notes: ^an (%); Median (IQR). ^bPearson's Chi-squared test; Wilcoxon rank sum test; Independent Samples t-test; *p*-value with FDR correction.

Nomogram Prediction Model Construction

Two distinct nomogram risk prediction models were developed and compared using the training dataset. Model 1, a full-variable model, was constructed via a two-stage variable selection process: initial screening with univariate logistic regression (FDR-corrected $p < 0.1$) followed by multivariate logistic regression minimizing the AIC. This yielded an optimal combination of 11 predictive indicators (AIC = 681.78), forming the basis for its nomogram (Figure 2a). Model 2 was a streamlined version built with the same methodology but incorporating only the four core predictive variables were statistically significant (FDR-corrected $p < 0.05$) in the multivariate logistic regression (Figure 2b). For both models, individual risk factor scores were summed to derive a total score, allowing for visual estimation of ASD early screening probability from a corresponding risk scale.

Nomogram Prediction Model Validation

We performed internal validation using Bootstrap resampling ($r = 5000$ iterations; random seed = 123 for reproducibility) to systematically evaluate both models' predictive performance. Model 1 exhibited strong discriminatory ability, with C-index values of 0.861 (95% CI: 0.825–0.893) for the training set and 0.865 (95% CI: 0.809–0.915) for the validation set. Calibration curves (Figures 3a and c) confirmed excellent agreement between observed and predicted values. ROC analysis yielded AUCs of 0.852 (95% CI: 0.817–0.885) and 0.845 (95% CI: 0.787–0.897) for the training and validation sets, respectively. Sensitivities were 77.5% and 78.6%, with specificities of 84.1% and 81.0% (Figure 3e).

Similarly, Model 2 also demonstrated strong discriminatory ability, with C-index values of 0.842 (95% CI: 0.805–0.878) for the training set and 0.824 (95% CI: 0.763–0.881) for the validation set. Calibration curves (Figures 3b and d) also showed good agreement. ROC analysis resulted in AUCs of 0.840 (95% CI: 0.801–0.874) for the training set and 0.819 (95% CI: 0.755–0.875) for the validation set. Sensitivities were 84.6% and 78.6%, with specificities of 73.4% and 79.0% (Figure 3f). A DeLong test comparing AUCs on the validation set showed no statistically significant difference between the two models ($p = 0.072$), indicating Model 2 was not inferior to Model 1. In summary, the two models do not have a significant difference in overall prediction performance, Model 2 maintained high predictive accuracy with fewer variables, enhancing its clinical utility and operability.

Discussion

This study leveraged logistic regression analysis to identify a comprehensive panel of blood markers in children with ASD, meticulously investigating their independent associations with ASD prevalence, and subsequently constructing a nomogram model for predicting ASD risk. Our findings not only revealed significant associations between peripheral blood markers and the risk of ASD onset, notably the non-linear “threshold effect” observed for calcium, but also successfully identified four independent blood-based risk factors crucial for the early identification and risk assessment of ASD: calcium, iron, vitamin D, and PDW. Model 2, a parsimonious model integrating these core variables, demonstrated commendable discriminative ability and predictive accuracy in the validation set (AUC = 0.819, C-index = 0.824),

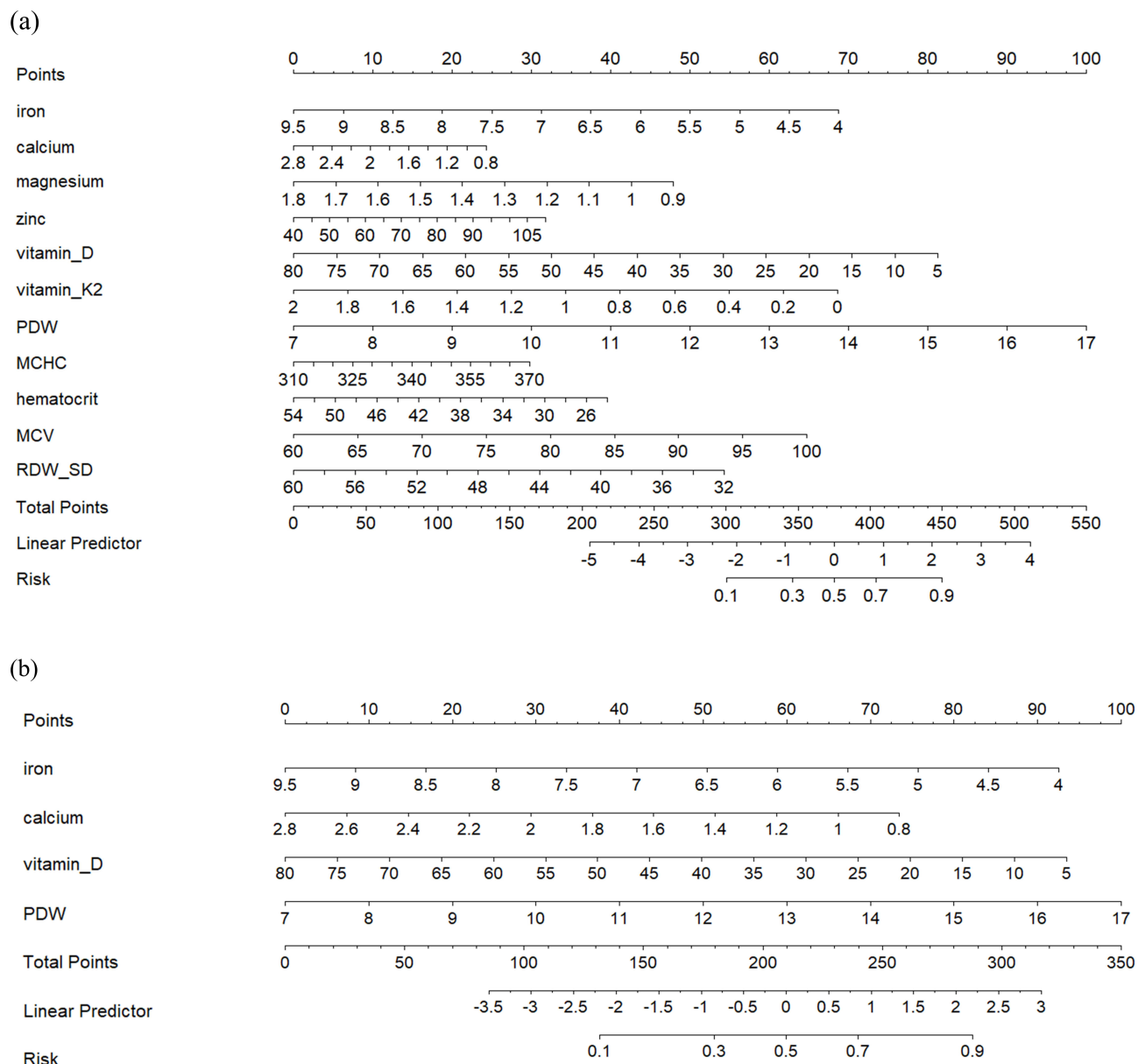


Figure 2 Nomogram Models for Predicting ASD Risk. (a) The nomogram for Model 1 predicts ASD risk using a combination of eleven blood-based variables. (b) The nomogram for Model 2 provides a simplified ASD risk prediction using four core independent risk factors.

achieving a favorable balance between sensitivity (78.6%) and specificity (79.0%). These results underscore its substantial potential for clinical utility in the early screening of ASD.

Calcium ions (Ca^{2+}) are critically involved in numerous neurodevelopmental processes, including neurotransmitter release, synaptic plasticity, and neuronal excitability.¹⁷ In this investigation, our RCS model elucidated a significant non-linear relationship between circulating calcium levels and ASD risk ($p < 0.001$). The non-linear dose-response curve identified a critical threshold of 1.13 mmol/L for serum calcium. Biologically, calcium ions are pivotal in synaptic plasticity and neuronal migration. The observed threshold effect suggests that calcium levels outside the optimal physiological range may disrupt these sensitive neurodevelopmental pathways. Specifically, lower blood calcium concentrations were significantly associated with an elevated ASD risk; when serum calcium levels fell below 1.13 mmol/L, the probability of ASD significantly increased. Conversely, as calcium levels surpassed 1.13 mmol/L, the risk gradually declined and subsequently stabilized. This observed phenomenon suggests a potential “threshold effect” for calcium levels in clinical intervention for ASD: insufficient calcium levels substantially amplify ASD risk,

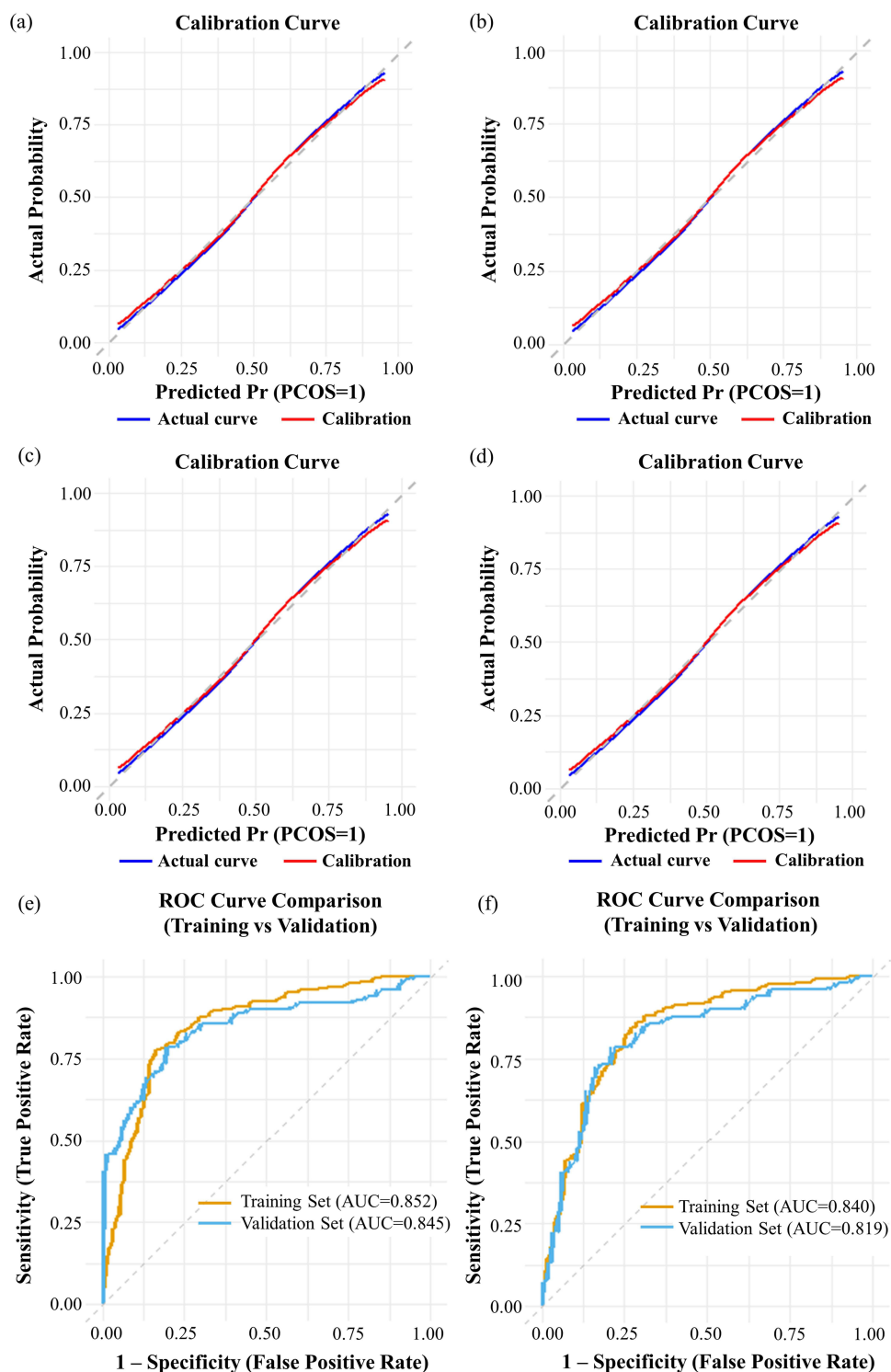


Figure 3 Validation and Performance of the Nomogram Prediction Models. (a) The calibration curve for Model 1 demonstrates the agreement between predicted and actual probabilities in the training set. (b) The calibration curve for Model 2 shows the model's performance on the training set. (c) The calibration curve for Model 1 is shown for the validation set to confirm its predictive accuracy. (d) The calibration curve for Model 2 demonstrates its performance on the validation set. (e) ROC curves compare the predictive performance of Model 1 in the training versus the validation datasets. (f) ROC curves illustrate the predictive accuracy of the simplified Model 2 in both the training and validation datasets.

whereas a moderate increase in calcium concentration contributes to risk reduction. A state of hypocalcemia can disrupt the delicate balance between excitatory glutamate and inhibitory gamma-aminobutyric acid (GABA), leading to neuronal hyperexcitability, which may underpin social deficits and repetitive behaviors characteristic of ASD.¹⁸ Furthermore, suboptimal calcium levels can impair mitochondrial function and augment the production of reactive oxygen species (ROS), thereby precipitating oxidative stress and neuroinflammation,¹⁹ all of which have been implicated in ASD pathophysiology. Nevertheless, it is crucial to note that excessively high blood calcium levels can also exert detrimental effects on the nervous system, as supernumerary calcium may induce neuronal damage through excitotoxicity.²⁰ Consequently, this finding provides a critical, targeted reference for nutritional interventions in ASD, strongly suggesting that an “individualized regulation” of calcium levels, rather than indiscriminate calcium supplementation, might be a more effective, actionable, and clinically meaningful approach.

We found a significant association between lower serum iron levels and a substantially increased risk of ASD (OR = 0.740, FDR-corrected $p = 0.021$). Iron is crucial for maintaining the integrity and function of the dopamine system; as a vital cofactor for tyrosine hydroxylase (TH), iron deficiency can lead to diminished dopamine synthesis, consequently impacting social abilities and emotional regulation.²¹ Conversely, iron deficiency may elevate levels of oxidative stress, compromising the brain's intrinsic antioxidant defense mechanisms, which in turn can contribute to neuroinflammation and synaptic dysfunction.^{22,23} Furthermore, as a fundamental component of myelin, iron deficiency might compromise white matter integrity in children with ASD, thereby impeding the efficient transmission of neural signals.^{24,25} Significantly lower serum iron levels in children with ASD compared to controls suggest that diminished iron bioavailability may be a predisposing risk factor for the disorder.

Vitamin D exerts broad-ranging effects on neurodevelopment, encompassing the regulation of brain calcium homeostasis, neurotransmitter synthesis, and immune modulation.²⁶ This study found that children with lower vitamin D levels had a significantly increased risk of ASD (OR = 0.973, FDR-corrected $p = 0.021$). Vitamin D possesses anti-inflammatory properties, capable of inhibiting excessive microglial activation and reducing the production of pro-inflammatory cytokines such as IL-6 and TNF- α , thereby ameliorating neuroinflammation.²⁷ Moreover, animal model studies have demonstrated that vitamin D deficiency can induce various alterations in brain synapses and neurotransmitter systems, abnormalities widely considered relevant to the core symptomatology of ASD.²⁸ Our study observed a significant disparity in vitamin D levels between children with ASD and neurotypical children, a finding consistent with previous reports by Mostafa and Al-Ayadhi, whose research indicated significantly lower serum vitamin D levels in children with ASD compared to healthy controls, with vitamin D levels negatively correlating with ASD severity scores (OR = 0.84).²⁹

We observed that children with ASD exhibited significantly higher PDW levels compared to healthy controls, and that an increase in PDW was significantly and positively correlated with ASD risk (OR = 1.290, $p < 0.001$). Platelets play a critical role in neuroinflammation; their release of various bioactive molecules, including platelet-derived growth factor (PDGF), chemokine CXCL4, and 5-hydroxytryptamine (5-HT), can exert profound influences on neurotransmitter regulation and synaptic function.³⁰ Furthermore, endothelial dysfunction is a commonly recognized pathological feature in children with ASD, wherein imbalances within the angiotensin system and elevated oxidative stress can augment platelet activity, consequently impacting cerebral microcirculation and potentially exacerbating the pathological progression of ASD.³¹ Thus, PDW could serve not only as a promising potential biomarker for ASD but also as a valuable indicator reflecting abnormal vascular and inflammatory states in children afflicted with the disorder.

The nomogram prediction model developed in this study demonstrated excellent predictive performance on the validation set, evidenced by a C-index of 0.824 and an AUC of 0.819. The high degree of fit observed on the calibration curve further indicates that the model not only possesses robust discriminatory ability but also exhibits commendable predictive accuracy and stability. Although the AUC of the full variable model (Model 1) was marginally higher at 0.845 compared to the core variable model (Model 2) at 0.819, a rigorous DeLong test revealed no statistically significant difference in predictive performance between the two models ($p = 0.072$). This crucial finding strongly supports our strategic selection of the core variable model. Given the absence of a significant difference in predictive efficacy, the core variable model's reliance on only four easily accessible peripheral blood markers significantly enhances its clinical utility and operational feasibility. This streamlined model mitigates increased complexity and cost, rendering it substantially

more amenable to widespread implementation in primary healthcare settings and routine physical examinations. This facilitates broad coverage and improved efficiency in ASD early screening. This strategic approach, which judiciously prioritizes model simplicity while steadfastly ensuring predictive accuracy, represents a significant paradigm shift in the development of contemporary medical prediction models and provides clinicians with a more practical and actionable tool for informed decision-making.

The model's practical utility is high, underscored by several key attributes. Its reliance on routine blood markers ensures easy implementation in standard pediatric care, and its intuitive nomogram allows for rapid, quantified risk assessment. The model demonstrates a balanced sensitivity (78.6%) and specificity (79.0%) in the validation set, signifying substantial screening utility. While we acknowledge that these metrics fall below the absolute highest performance reported in the literature, it is crucial to contextualize our model within the current landscape of ASD prediction. Models employing advanced methodologies, such as structural and functional neuroimaging or polygenic risk scores,^{32,33} often achieve higher AUC values but are inherently constrained by high cost and non-scalability, rendering them unsuitable for routine, large-scale clinical screening. Furthermore, while other studies have utilized objective biomarkers via complex metabolomic or proteomic platforms,^{34,35} these markers are not routinely available in standard hospital laboratories.

In contrast, our nomogram is deliberately built upon four routine, low-cost blood indicators. Our achieved performance (AUC=0.819) demonstrates a strong discriminative ability, representing a significant cost-effectiveness advantage over complex models. Moreover, our model offers an objective, quantitative assessment that complements existing subjective behavioral screening tools. Since behavioral screeners can suffer from variable specificity and reliance on parental report, a robust blood-based tool utilizing commonly measured markers provides a valuable first-tier objective risk stratification mechanism, promoting early and targeted diagnostic evaluation without significant logistical burden. Rigorous validation using independent training and validation sets further enhances its credibility as a tool for early ASD risk identification and for guiding personalized interventions.

Our final predictive model, consisting of calcium, iron, vitamin D, and PDW, demonstrated superior predictive power and parsimony. It is notable that certain biomarkers frequently studied in ASD research, such as zinc and magnesium, did not meet the statistical criteria for independent prediction in our final multivariate model. While several systematic reviews and meta-analyses suggest an overall tendency for lower zinc levels in some ASD cohorts,^{36,37} and others report abnormalities in magnesium,³⁸ the literature surrounding these specific micronutrients is characterized by significant heterogeneity and inconsistency. Indeed, studies often report conflicting findings concerning zinc and magnesium levels. This disparity is likely influenced by key methodological differences, including sample matrix (serum, whole blood, hair, or urine), age and geographic location of the cohort, as well as variations in dietary intake and measurement techniques.^{39,40} For instance, large cohort studies have not consistently identified a significant association between neonatal magnesium levels and subsequent ASD risk.⁴¹ Therefore, the exclusion of zinc and magnesium from our data-driven nomogram, despite their biological relevance, underscores a critical point: the independent predictive power of a biomarker must be confirmed within the specific statistical framework and cohort under investigation. Our rigorous selection process prioritized those markers that demonstrated robust, non-redundant predictive value in our population.

As a cross-sectional study, we cannot infer definitive causality; the observed nutrient deficiencies might be secondary consequences (reverse causality) of ASD-related behaviors, such as selective eating patterns⁴² or gastrointestinal dysfunction. Nevertheless, our nomogram retains significant clinical utility as an early screening tool. Regardless of the causal direction, these biomarkers effectively identify high-risk individuals based on their current biochemical profiles, serving as a valuable first-tier objective assessment in primary care. Future prospective longitudinal studies are essential to definitively establish the temporal sequencing and causal links between these markers and ASD onset.

While this study offers valuable insights into potential blood biomarkers for early ASD screening, it's crucial to acknowledge its limitations. Primarily, A key methodological limitation is the reliance on PSM solely for age and sex. While PSM effectively mitigates confounding from these measured variables, it cannot account for unmeasured confounders, such as socioeconomic status, environmental exposures, or specific perinatal factors, which may still influence the observed biomarker associations. Second, the retrospective design and reliance solely on internal validation

necessitate cautious interpretation. Although techniques like training/validation set partitioning and the Bootstrap method confirm the model's stability within the existing data, its generalizability to independent, external datasets remains unconfirmed. This is particularly relevant given the single-center origin of our sample, which introduces the potential for regional bias. Therefore, future multi-center, large-sample prospective cohort studies are essential to rigorously validate the predictive utility and investigate potential causal relationships of these markers across diverse pediatric populations, ensuring the model's broad applicability and clinical translational potential.

Conclusion

This study furnishes valuable information regarding potential biomarkers for the early screening of ASD, having successfully constructed and validated a nomogram model for ASD risk based on circulating levels of iron, calcium, vitamin D, and PDW. This model directly addresses the unmet need for objective biomarkers in ASD, offering critical insights for early identification, acquired assessment, and precise intervention strategies. Furthermore, it lays a robust foundation for the future development of blood marker-based approaches for both early ASD screening and the implementation of individualized risk assessment paradigms.

Data Sharing Statement

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

Ethics Approval

The study was conducted in accordance with the Declaration of Helsinki, and approved by The Ethics Committee of Dalian Women and Children's Medical Group granted approval for this study (Approval No.: DUTBME231128-01).

Author Contributions

Lin Lin and Xixi Wang are co-first authors. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have declared that no competing interest exists.

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