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Associations between amino acid levels and autism spectrum disorder severity

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Abstract

Background Autism spectrum disorder (ASD) imposes a significant burden on both patients and society. Amino acid metabolism abnormalities are particularly relevant to ASD pathology due to their crucial role in neurotransmitter synthesis, synaptic function, and overall neurodevelopment. This study aims to explore the association between amino acid metabolic abnormalities and the severity of ASD by analyzing the amino acid concentrations in the blood of children with ASD.

Methods Fasting peripheral blood samples were collected from 344 children with ASD, and amino acid concentrations were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) while strictly following quality control measures. The association between amino acid concentrations and ASD severity was evaluated using logistic regression and restricted cubic spline (RCS) analysis. The ROC (receiver operating characteristic) curve, decision curve analysis (DCA), and calibration curve were used to construct and validate predictive models and nomograms, thereby assessing their predictive performance.

Results Multivariate logistic regression analysis showed that aspartic acid (OR = 1.037, 95% CI: 1.009–1.068, $P = 0.01$), glutamic acid (OR = 1.009, 95% CI: 1.001–1.017, $P = 0.03$), phenylalanine (OR = 1.036, 95% CI: 1.003–1.072, $P = 0.04$), and leucine/isoleucine (OR = 1.021, 95% CI: 1.006–1.039, $P = 0.01$) were significantly positively correlated with the severity of ASD. On the other hand, tryptophan (OR = 0.935, 95% CI: 0.903–0.965, $P < 0.01$) and valine (OR = 0.987, 95% CI: 0.977–0.997, $P = 0.01$) were significantly negatively correlated with the severity of ASD. RCS analysis further revealed a nonlinear relationship between the concentrations of aspartic acid, proline, and glutamic acid and the risk of ASD. ROC curve analysis showed that the combined model achieved an AUC (area under the curve) of 0.806, indicating high diagnostic accuracy. Calibration and decision curve analysis further validated the predictive effectiveness and clinical utility of the model.

Conclusions This study identifies potential amino acid biomarkers that may contribute to ASD severity assessment. Further research is needed to validate these findings and explore their clinical utility.

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Keywords Autism spectrum disorder, Amino acid metabolism, Glutamic acid, Risk assessment, Restricted cubic spline analysis, Early diagnosis

Introduction

Autism Spectrum Disorder (ASD) is a pervasive neurodevelopmental disorder that typically manifests in early childhood and persists throughout life. The core features of ASD include significant deficits in social interaction and communication, as well as repetitive behaviors and restricted interests. Additionally, many individuals with ASD present with sensory processing abnormalities, intellectual disability, anxiety, attention deficits, and epilepsy [1]. These symptoms not only severely impact the quality of life and social functioning of affected individuals but also impose a heavy burden on families, educational systems, and societal resources.

Amino acids are central molecules in various metabolic pathways in the human body, playing important physiological and pathological roles [2]. They are not only the basic building blocks of proteins but also participate in the synthesis and regulation of neurotransmitters, maintenance of immune function, regulation of redox reactions, nitrogen balance, and gene expression [3]. Investigations into metabolic disturbances in ASD have revealed significant associations with altered amino acid profiles. A research has demonstrated that mutations in the BCKDK gene, which regulates branched-chain amino acid metabolism, can lead to autism with comorbid epilepsy and intellectual disability [4]. This connection between amino acid metabolic pathways and neurodevelopmental outcomes is further supported by studies showing how inborn errors of metabolism can significantly impact brain function and development [5, 6]. A deeper understanding of the role of amino acids in the pathogenesis of ASD could contribute to the development of more effective diagnostic and therapeutic strategies, thereby reducing the overall burden on patients and society. We hypothesize that altered amino acid metabolism is associated with ASD severity and that specific amino acids could serve as potential biomarkers for ASD risk assessment.

In recent years, research on the relationship between amino acid metabolism and ASD has increased significantly. Studies have found that reduced levels of ornithine and elevated levels of valine may increase the risk of ASD by interfering with the metabolic pathway of the nickel transport system [7]. Further research has shown that tryptophan levels in individuals with ASD are typically significantly lower, directly leading to insufficient serotonin synthesis [8]. Serotonin, a neurotransmitter crucial for mood, behavior, and cognitive function, is believed to be a key factor in the emotional and behavioral regulation problems observed in ASD patients. Tryptophan is the sole precursor for serotonin synthesis, and its depletion can disrupt the serotonergic system, which regulates numerous neurodevelopmental processes including neurogenesis, synaptogenesis, and neural circuit formation critical for social behavior and emotional regulation [9, 10]. Moreover, glutamate, a key excitatory neurotransmitter in the brain, is closely associated with excitotoxicity through overactivation of N-methyl-D-aspartate (NMDA) receptors, which plays a significant role in the neurodevelopmental abnormalities seen in ASD [11]. Glutamic acid and its related metabolic pathways are fundamental to proper brain development and function. Excessive glutamate signaling can disrupt the excitatory-inhibitory balance in neural circuits, leading to abnormal synaptic plasticity and connectivity patterns observed in ASD [12, 13]. Similarly, aspartic acid, another excitatory amino acid, functions as a neurotransmitter and participates in the urea cycle and gluconeogenesis. Alterations in aspartic acid levels can affect neurotransmission and energy metabolism in neurons, potentially contributing to the neurobiological basis of ASD [14]. Imbalance in glutamatergic neurotransmission, whether due to elevated glutamate levels or impaired regulatory mechanisms, can lead to excitotoxicity, causing neuronal damage [15]. This neuronal damage is closely linked to the cognitive and behavioral symptoms observed in individuals with ASD. Studies have also found that concentrations of total branched-chain amino acids (BCAAs), valine, and leucine/isoleucine are higher in children with ASD. Even after adjusting for potential confounding factors such as age, gender, and body mass index, these amino acids remain positively correlated with ASD risk [16]. BCAAs play crucial roles in protein synthesis, energy production, and as precursors for neurotransmitters. They compete with aromatic amino acids for transport across the blood-brain barrier, potentially affecting the synthesis of neurotransmitters like dopamine and serotonin, which are implicated in ASD pathophysiology [17, 18]. These findings suggest that abnormalities in amino acid metabolism may play a critical role in the pathogenesis of ASD.

Our study aims to investigate the relationship between amino acid metabolic profiles and ASD severity in children with ASD. The aim was to provide new insights into the pathogenesis of ASD and to offer scientific evidence for the development of clinical intervention strategies. Ultimately, this research sought to explore the role of amino acid metabolic abnormalities in ASD, identify potential biomarkers, and provide theoretical support for early diagnosis and personalized treatment of ASD.

Methods and materials

Study participants

The study involved children diagnosed with ASD who were admitted to the Pediatrics Department at the First Affiliated Hospital of Henan University of Chinese Medicine. Based on epidemiological studies in China, the prevalence of ASD in the 0–14 age group is approximately 0.4%, with a slightly lower rate of 0.3% among children aged 7–14 years. Given this epidemiological context, participants were selected with an inclusive age range to comprehensively capture metabolic characteristics across critical developmental stages [19]. Participants were selected based on several criteria including being between 4 months and 12 years old, meeting the DSM-5 diagnostic criteria for autism, having complete clinical data such as age, gender, and Childhood Autism Rating Scale (CARS) scores, and obtaining informed consent from parents or legal guardians [20]. Children were excluded if they had other organic brain diseases, severe heart, liver, or kidney conditions, or had used antibiotics, probiotics, or prebiotics within four weeks prior to sample collection.

Sample collection

Peripheral blood samples were collected from all study subjects in a fasting state early in the morning. The blood was dripped onto specialized filter paper for blood collection, with each blood spot having a diameter of ≥ 8 mm, ensuring the samples were uncontaminated and air-dried naturally. The samples were then sent to Beijing Fuyou Longhui Genetic Specialty Outpatient Clinic for amino acid analysis.

Amino acid detection

Amino acid detection was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The process involved cutting the dried blood filter paper into circular spots with a diameter of 3 mm (equivalent to 3.2 μl of whole blood) using a puncher. These spots were then placed into 0.6 ml centrifuge tubes for processing. An extraction solvent containing an isotope-labeled internal standard was added to the tubes for extraction. After drying the extract under nitrogen, a derivatization reagent was introduced for the derivatization process. Once this step was completed, the samples were dried again, followed by the addition of a mobile phase reconstitution solution to prepare them for subsequent analysis. Amino acid assays include lysine, alanine, aspartic acid, glutamic acid, methionine, phenylalanine, tyrosine, leucine/isoleucine, tryptophan, valine, citrulline, arginine, glycine, ornithine, histidine, serine, threonine and proline.

Sample quality control

To ensure the reliability of the experiment, strict quality control measures were implemented throughout the study. Before sample analysis, laboratory sample reception personnel inspected each sample according to the "Technical Specifications for Blood Spot Collection for Neonatal Genetic Metabolic Disease Screening." Only samples that met the specifications were processed for detection; those that did not meet the requirements were returned for recollection. During sample analysis, the testing environment was monitored and controlled to ensure compliance with the methods and equipment requirements, and reagents and consumables that passed quality checks were used. Quality control samples were measured simultaneously with each batch of samples, and quality control charts were created. Detection reports were issued only when quality control standards were met. Additionally, the laboratory participates annually in the inter-laboratory quality assessment program organized by the CDC, including three PT tests and two QC tests each year, all of which have yielded satisfactory results, further validating the reliability of the detection methods.

Statistical analysis

To investigate the relationship between amino acid concentrations and the severity of ASD, this study defined severe ASD as a CARS score > 36 and mild/moderate ASD as a CARS score ≤ 36. Descriptive analysis was performed on all variables, with data primarily presented as medians (interquartile ranges). In descriptive statistics, comparisons of continuous variables were performed using Mann-Whitney U tests, and binary variables were analyzed using the chi-square test.

Univariate logistic regression analysis was conducted on 19 amino acids (including lysine, alanine, aspartic acid, glutamic acid, methionine, phenylalanine, tyrosine, leucine/isoleucine, tryptophan, valine, citrulline, arginine, glycine, ornithine, histidine, serine, threonine, and proline), as well as gender and age variables. Regression coefficients, *P*-values, ORs, and 95% confidence intervals were calculated to analyze the associations between these variables and the severity of ASD.

Spearman correlation tests were used to evaluate the correlations between amino acid concentrations and CARS scores, as well as the interrelationships among these amino acids. In the multivariate logistic regression analysis, to eliminate collinearity among variables, we selected variables with a variance inflation factor (VIF) less than 5 and constructed a multivariate logistic regression model based on these variables. Model 1 included gender and age, while Model 2 adjusted for gender, age adn BMI to ensure the robustness of the analysis results.

Given the diversity of amino acids, we selected statistically significant variables from the multivariate logistic regression analysis for further study. We employed several advanced statistical techniques to comprehensively evaluate the relationship between amino acid concentrations and ASD severity. Restricted cubic spline (RCS) analysis was utilized to capture the potential nonlinear relationships between amino acids and ASD severity, which traditional linear models might fail to detect. Unlike linear regression that assumes a constant effect across the entire range of a variable, RCS allows for more flexible and complex associations by using a series of joined line segments (nodes) at the 25th, 50th, and 75th percentiles of the variable distribution, with the 10th percentile as the reference node [8]. This approach enables a more nuanced understanding of how amino acid concentrations might differentially impact ASD severity at various concentration levels [16]. To rigorously assess the predictive performance, we conducted receiver operating characteristic (ROC) curve analysis to evaluate the discriminatory power of individual amino acids and the multivariable model. The area under the ROC curve (AUC) provides a comprehensive measure of the model's ability to distinguish between different ASD severity levels.

Decision curve analysis (DCA) was employed to translate statistical performance into potential clinical utility. Unlike traditional metrics that focus solely on statistical significance, DCA assesses the net benefit of a predictive model across different clinical decision thresholds. This method allows clinicians to understand the practical value of the amino acid profile in making clinical decisions, considering the potential risks and benefits of intervention at various probability thresholds. Calibration curves were used to validate the model's predictive accuracy by comparing predicted probabilities with observed outcomes. Finally, we constructed a nomogram to provide a visual and intuitive representation of how each amino acid contributes to predicting ASD severity, facilitating easier interpretation of the complex multivariate relationships.

All statistical analyses were conducted using R software (version 4.4.0). For all statistical tests, a two-tailed P-value < 0.05 was considered statistically significant.

Results

A total of 344 participants were involved in this study, of which 287 were male (83.4%) and 57 were female (16.6%). The baseline characteristics of the participants are summarized in Table 1. The overall median CARS score

Table 1 General characteristics of the study participants

Variable	Overall ($n=344$)	Mild/Moderate ($n=62$)	Severe ($n=282$)	*P*-value
CARS_Score	41.00 (37.00, 44.00)	34.00 (32.00, 34.00)	42.00 (39.00, 44.00)	< 0.001
Gender				0.155
Male, *n* (%)	287 (83.43)	56 (90.32)	231 (81.91)	
Female, *n* (%)	57 (16.57)	6 (9.68)	51 (18.09)	
Age	3.00 (2.00, 4.00)	3.00 (3.00, 4.75)	3.00 (2.00, 4.00)	0.004
BMI, kg/m^2	16.15 (14.97, 17.31)	16.12 (15.01, 17.36)	16.15 (14.90, 17.30)	0.968
Amino acid (μmol/L)				
Lysine	77.84 (58.15, 109.13)	85.62 (68.34, 115.02)	75.38 (56.69, 106.80)	0.033
Methionine	191.04 (152.91, 237.56)	187.98 (145.02, 270.37)	191.43 (154.03, 234.11)	0.855
Phenylalanine	47.97 (40.38, 60.11)	43.00 (32.95, 53.96)	49.21 (41.50, 60.59)	< 0.001
Tryptophan	186.15 (149.68, 230.79)	159.31 (131.23, 219.67)	189.42 (154.48, 232.91)	0.017
Leucine/Isoleucine	18.88 (15.58, 23.39)	20.43 (15.48, 22.96)	18.52 (15.61, 23.43)	0.317
Valine	65.37 (53.96, 76.99)	58.25 (46.37, 70.78)	66.30 (55.18, 78.84)	0.005
Threonine	56.20 (45.82, 68.39)	51.33 (44.49, 67.92)	57.17 (45.86, 68.58)	0.35
Histidine	134.56 (109.36, 162.49)	123.96 (104.11, 155.72)	137.95 (112.11, 164.78)	0.022
Alanine	34.76 (26.40, 45.73)	39.45 (31.62, 49.08)	33.51 (25.69, 45.03)	0.003
Aspartic	155.88 (130.03, 189.38)	162.83 (134.06, 197.18)	153.71 (129.53, 186.12)	0.386
Glutamic	10.98 (9.43, 13.36)	10.88 (9.52, 12.68)	11.04 (9.27, 13.60)	0.76
Tyrosine	4.21 (2.16, 7.13)	4.58 (2.98, 6.87)	4.12 (2.11, 7.25)	0.256
Citrulline	233.71 (180.07, 303.75)	232.77 (182.81, 314.93)	234.34 (179.76, 299.65)	0.715
Arginine	44.91 (34.94, 63.73)	50.39 (38.02, 73.19)	44.17 (34.55, 60.97)	0.06
Glycine	84.40 (61.30, 113.78)	83.33 (61.79, 118.57)	85.03 (61.31, 109.70)	0.764
Ornithine	114.59 (88.65, 144.82)	110.37 (86.64, 143.60)	114.98 (89.25, 145.25)	0.657
Serine	37.86 (29.93, 49.76)	37.59 (30.62, 50.86)	38.55 (29.65, 49.22)	0.626
Proline	409.07 (297.26, 590.89)	439.02 (330.43, 746.33)	404.18 (296.05, 561.98)	0.293

For continuous variables (CARS Score, Age, BMI, and amino acid concentrations), we used the Mann-Whitney U test

For categorical variables (gender, *n* (%)), we used Chi-square tests

was 41.00, with the median score for the mild/moderate autism group being 34.00, and 42.00 for the severe autism group, indicating a significant difference between the two groups ($P < 0.05$). Further analysis revealed that certain amino acids, such as phenylalanine, tryptophan, leucine/isoleucine, aspartic acid, and glutamic acid, showed significant differences between the mild/moderate autism group and the severe autism group. This suggests that the levels of these amino acids may be associated with the severity of autism. However, the levels of other amino acids did not show significant differences between the two groups.

In the univariate analysis, a separate logistic regression analysis was performed for each amino acid variable to assess its independent effect on the severity of ASD (Fig. 1). The results showed that age (OR = 0.833, 95% CI: 0.732–0.950, $P < 0.05$) was significantly negatively correlated with the severity of ASD, indicating that with increasing age, the risk of severe ASD decreases by approximately 16.7%. Although the effect of gender did not reach statistical significance (OR = 2.061, 95% CI: 0.903–5.574, $P = 0.11$), it suggests that females may face a higher risk of severe ASD. Aspartic acid (OR = 1.035, 95% CI: 1.014–1.059, $P < 0.05$) and glutamic acid (OR = 1.005, 95% CI: 1.000-1.010, $P = 0.045$) were significantly positively correlated with ASD severity, indicating that increases in the levels of these amino acids were associated with approximately 3.5% and 0.5% increases in risk, respectively. Phenylalanine (OR = 1.018, 95% CI: 1.003–1.035, $P = 0.03$) and leucine/isoleucine (OR = 1.008, 95% CI: 1.001–1.016, $P = 0.03$) were also significantly associated with increased risk, leading to approximately 1.8% and 0.8% increases in risk, respectively. On the other hand, tryptophan (OR = 0.982, 95% CI: 0.966–0.997, $P = 0.02$) was significantly negatively correlated with the severity of ASD, with its increased levels reducing the risk by approximately 1.8%. Other amino acids did not show significant associations with the severity of ASD. Figure S1 shows the correlations between CARS scores and amino acid concentrations, as well as inter-amino acid correlations. Table S1 presents the variance inflation factors for all amino acids, confirming that multicollinearity was not a concern (all VIFs < 5) for our regression analyses.

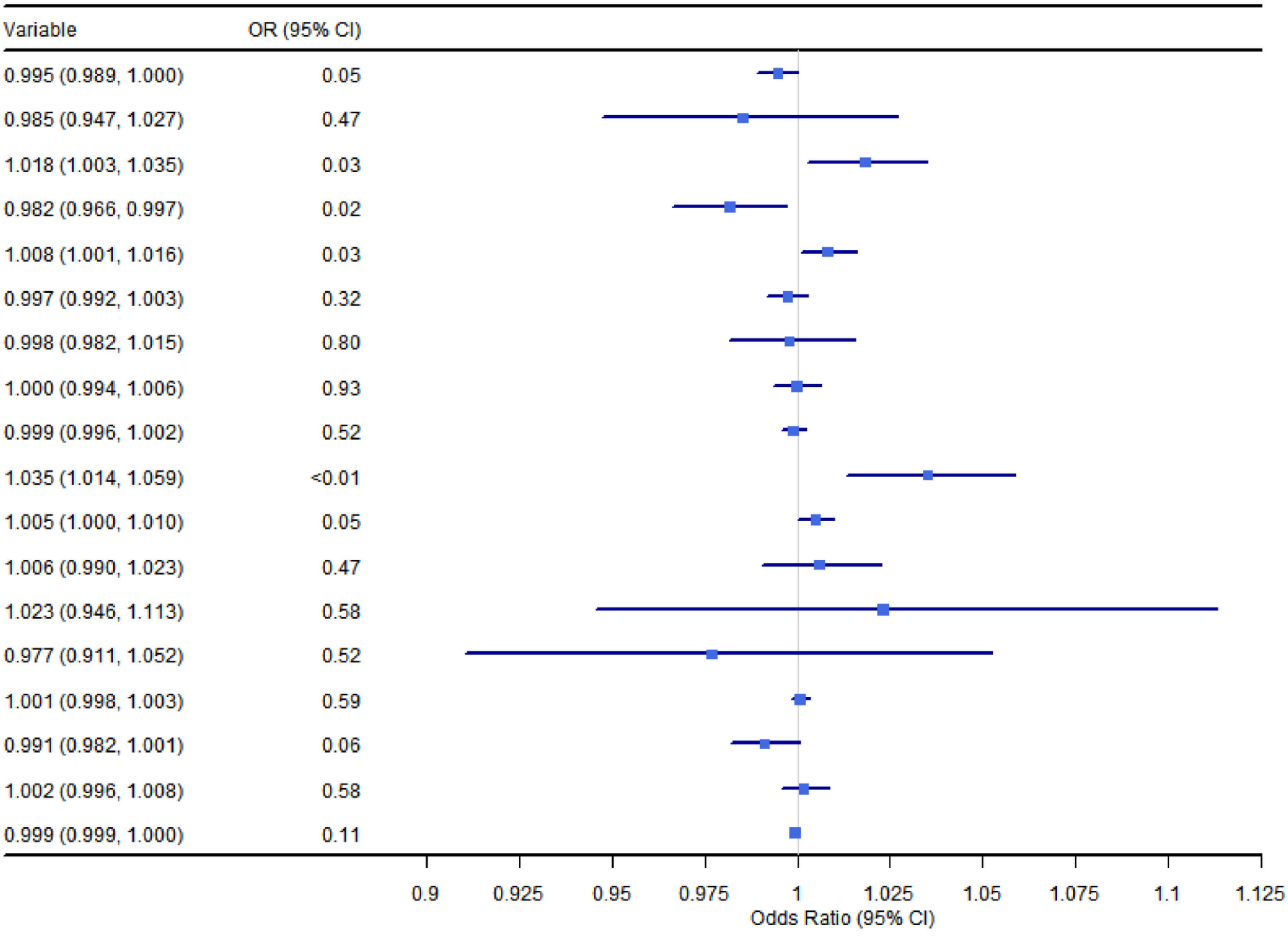

Fig. 1 Univariate logistic regression analysis: the association between amino acid concentrations, age, gender, and the severity of ASD

In the multivariate logistic regression analysis, we evaluated the independent effects of several variables on the severity of ASD (Table 2). The results showed that age (OR = 0.758, 95% CI: 0.636–0.899, $P < 0.01$) was significantly negatively correlated with ASD severity, indicating that the risk of severe ASD decreases with increasing age. Regarding amino acids, aspartic acid (OR = 1.037, 95% CI: 1.009–1.068, $P = 0.01$), glutamic acid (OR = 1.009, 95% CI: 1.001–1.017, $P = 0.03$), phenylalanine (OR = 1.036, 95% CI: 1.003–1.072, $P = 0.03$), and leucine/isoleucine (OR = 1.021, 95% CI: 1.006–1.038, $P = 0.01$) were significantly positively correlated with ASD severity. On the other hand, tryptophan (OR = 0.935, 95% CI: 0.903–0.965, $P < 0.01$) and valine (OR = 0.987, 95% CI: 0.977–0.997, $P = 0.01$) were significantly negatively correlated with ASD severity. These results indicate that specific amino acids play a significant role in the severity of ASD, maintaining their independent effects even after adjusting for age and gender.

According to the results of the RCS regression analysis, various amino acid concentrations exhibited different degrees of nonlinear correlations with the risk of ASD (Fig. 2). Phenylalanine (overall $P < 0.01$, nonlinear $P < 0.06$), aspartic acid (overall $P < 0.0001$, nonlinear $P < 0.01$), and proline (overall $P < 0.04$, nonlinear $P < 0.05$) all showed a nonlinear relationship with ASD risk, characterized by an initial increase followed by a decrease. Tryptophan (overall $P < 0.02$, nonlinear $P < 0.27$) was associated with an increased risk at low concentrations and a decreased risk at high concentrations. Leucine/isoleucine (overall $P < 0.03$, nonlinear $P < 0.27$) and glutamic acid (overall $P < 0.005$, nonlinear $P < 0.02$) displayed fluctuating risk patterns. Valine (overall $P < 0.68$, nonlinear $P < 0.75$) showed a trend of decreased risk at low concentrations and increased risk at high concentrations. Ornithine (overall $P < 0.55$, nonlinear $P < 0.999$) did not show a significant nonlinear relationship. These results suggest that changes in the concentrations of specific amino acids are associated with a complex nonlinear relationship to the risk of developing ASD, warranting further investigation into the underlying mechanisms and clinical significance (Fig. 2).

The nomogram illustrates a predictive model integrating multiple variables to estimate the probability of severe ASD among patients with ASD (Fig. 3B). According to the ROC curve analysis, the cutoff level for Aspartic Acid was 44.36 μmol/L with an AUC of 0.64; for Glutamic Acid, the cutoff level was 157.91 μmol/L with an AUC of 0.60; for Phenylalanine, the cutoff level was 50.44 μmol/L with an AUC of 0.62; and for Leucine Isoleucine and Tryptophan, the cutoff levels were 127.11 μmol/L and 35.10 μmol/L, respectively, with AUCs of 0.60 and 0.62 (Fig. 3A; Table 3). These variables demonstrated moderate performance in differentiating ASD severity, with P-values indicating statistical significance ($P < 0.05$). In contrast, the AUCs for Valine, Ornithine, and Proline

Table 2 Odds ratios (95% CI) of amino acid aoncentrations, age, gender and BMI with the severity of ASD

Variable	Model 1		Model 2	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
BMI			1.011 (0.895, 1.153)	0.87
Age	0.760 (0.640, 0.899)	< 0.01	0.758 (0.636, 0.899)	< 0.01
Gender	2.183 (0.836, 6.588)	0.13	2.156 (0.821, 6.575)	0.14
Lysine	0.994 (0.985, 1.003)	0.19	0.994 (0.985, 1.003)	0.18
Methionine	0.944 (0.884, 1.008)	0.08	0.944 (0.884, 1.008)	0.09
Phenylalanine	1.036 (1.003, 1.072)	0.03	1.036 (1.003, 1.072)	0.04
Tryptophan	0.935 (0.904, 0.965)	< 0.01	0.935 (0.903, 0.965)	< 0.01
Leucine_Isoleucine	1.021 (1.006, 1.038)	0.01	1.021 (1.006, 1.038)	0.01
Valine	0.987 (0.977, 0.997)	0.01	0.987 (0.977, 0.997)	0.01
Threonine	1.024 (0.991, 1.060)	0.16	1.024 (0.991, 1.060)	0.16
Histidine	1.004 (0.993, 1.015)	0.54	1.004 (0.993, 1.015)	0.54
Alanine	0.999 (0.993, 1.006)	0.83	0.999 (0.993, 1.006)	0.82
Aspartic_Acid	1.037 (1.009, 1.068)	0.01	1.037 (1.009, 1.068)	0.01
Glutamic_Acid	1.009 (1.001, 1.017)	0.03	1.009 (1.001, 1.017)	0.03
Tyrosine	1.012 (0.988, 1.039)	0.33	1.013 (0.988, 1.039)	0.32
Citrulline	1.018 (0.910, 1.148)	0.77	1.017 (0.909, 1.147)	0.78
Arginine	1.092 (0.976, 1.228)	0.13	1.091 (0.976, 1.228)	0.13
Glycine	0.997 (0.992, 1.002)	0.28	0.997 (0.992, 1.002)	0.28
Ornithine	0.984 (0.970, 0.998)	0.03	0.984 (0.970, 0.998)	0.03
Serine	1.001 (0.989, 1.013)	0.93	1.001 (0.989, 1.013)	0.92
Proline	0.999 (0.998, 1.000)	0.05	0.999 (0.998, 1.000)	0.05

Model 1, adjusted for age, gender;

Model 2, adjusted for age, gender, BMI

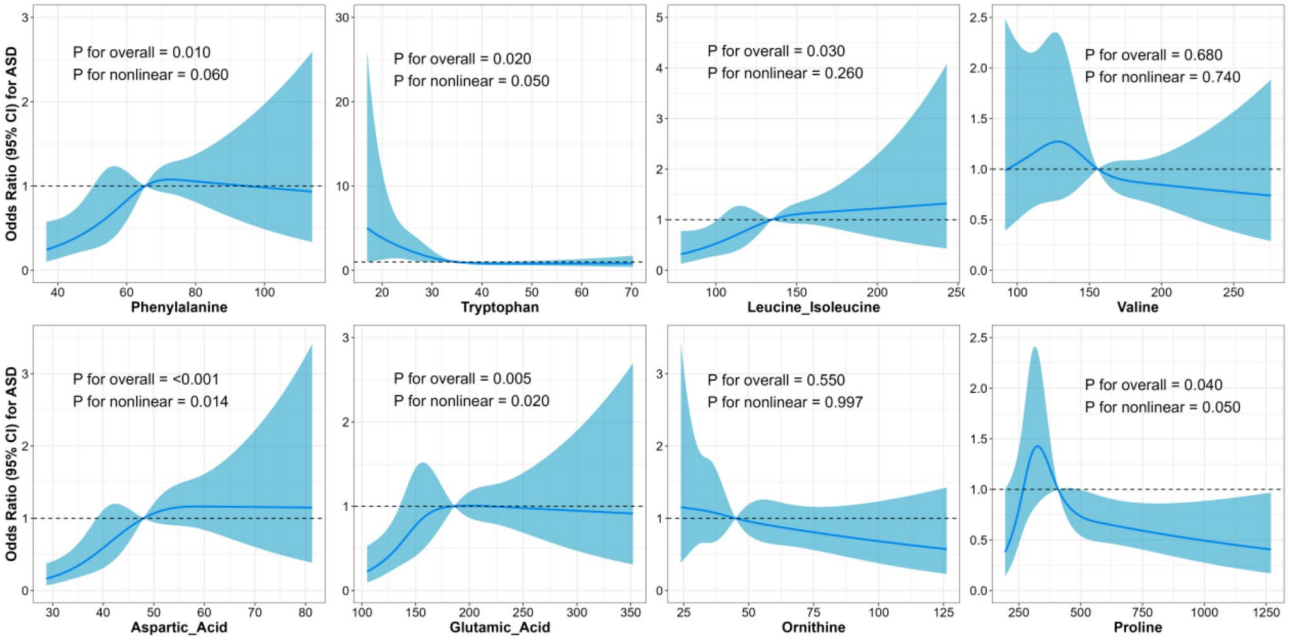

Fig. 2 The nonlinear relationship between amino acid concentrations and the severity of ASD (Phenylalanine, Tryptophan, Leucine/Isoleucine, Valine, Aspartic Acid, Glutamic Acid, Ornithine, and Proline)

were 0.54, 0.58, and 0.55, respectively, indicating lower performance in severity stratification, and their P-values were not statistically significant. Overall, the combined model achieved an AUC of 0.806, which was significantly higher than any single variable, demonstrating greater accuracy in distinguishing between mild/moderate and severe ASD cases (Fig. 3A; Table 3). Calibration curve analysis further validated the predictive capability of the model, showing a high concordance between the predicted probabilities and actual observations, with an average absolute error of only 0.02 (Fig. 3C). Decision curve analysis indicated that, compared to strategies assuming all or no individuals have severe ASD, using this model provided higher net benefits across a wide range of risk threshold probabilities (0.1 to 0.8), demonstrating its potential clinical value in stratifying ASD severity (Fig. 3D).

Discussion

This study aims to explore the correlation between amino acid concentrations and the severity of ASD in children. Through the analysis of various amino acids, it was found that aspartic acid, glutamic acid, phenylalanine, and leucine/isoleucine are positively correlated with ASD severity, while tryptophan and valine are significantly negatively correlated with ASD severity. These findings deepen our understanding of the underlying physiological mechanisms of ASD.

In recent years, research has increasingly focused on the association between amino acids and ASD in children, but conclusions are often inconsistent due to limited sample sizes or participant heterogeneity [21]. Our study, with its relatively large sample size ($n = 344$), provides an opportunity to examine these relationships more robustly. Regarding demographic factors, our analysis revealed an interesting negative correlation between age and ASD severity. This association suggests that older children in our sample tended to present with less severe ASD symptoms compared to younger children. However, we did not specifically control for developmental level, cognitive functioning, or intervention history—factors that may confound the relationship between age and symptom severity. The Nomogram we developed showed better predictive value for children over 3 years old, which aligns with other studies that have observed potential changes in symptom presentation across different developmental stages [22].

In terms of amino acids, this study found that aspartic acid, glutamic acid, phenylalanine, and leucine/isoleucine were significantly positively correlated with the severity of ASD. These amino acids play key roles in the nervous system, especially glutamic acid and aspartic acid, which are the main excitatory neurotransmitters involved in many important physiological processes [23, 24]. Glutamic acid is one of the most important excitatory neurotransmitters in the central nervous system, extensively involved in the regulation of learning, memory, and neural plasticity [25]. By activating NMDA receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, glutamic acid promotes signal transmission between neurons [26]. However, excessive release of glutamic acid may lead to over-excitation of

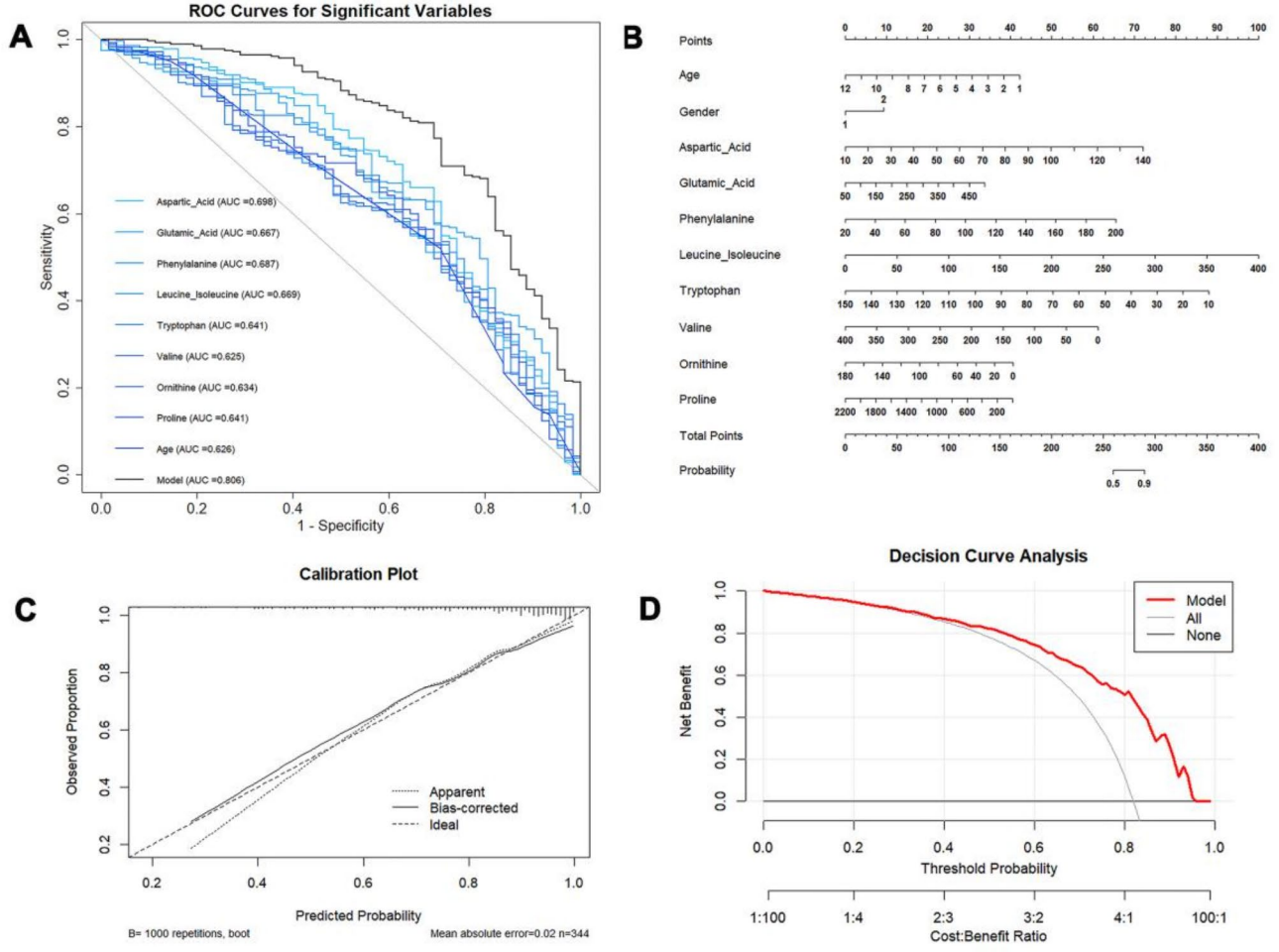

Fig. 3 ASD severity prediction model based on multivariable analysis: ROC Curve, Nomogram, Calibration Plot, and Decision Curve Analysis (Phenylalanine, Tryptophan, Leucine/Isoleucine, Valine, Aspartic Acid, Glutamic Acid, Ornithine, and Proline)

Table 3 Thresholds, AUC, sensitivity, and specificity of different amino acid concentrations and age in predicting ASD severity in multivariable analysis (Phenylalanine, Tryptophan, leucine/isoleucine, Valine, aspartic acid, glutamic acid, ornithine, and Proline)

Variable	Threshold	AUC	Sensitivity	Specificity	ppv	npv	*P_value*
Aspartic_Acid	44.36	0.64 (0.56, 0.73)	67.38	56.45	87.56	27.56	< 0.01
Glutamic_Acid	157.91	0.60 (0.52, 0.69)	72.34	50.00	86.81	28.44	0.03
Phenylalanine	50.44	0.62 (0.54, 0.70)	85.11	35.48	85.71	34.38	0.02
Leucine_Isoleucine	127.11	0.60 (0.52, 0.68)	60.28	59.68	87.18	24.83	0.02
Tryptophan	35.10	0.62 (0.55, 0.69)	56.38	70.97	89.83	26.35	0.02
Valine	169.84	0.54 (0.46, 0.62)	64.54	45.16	84.26	21.88	0.34
Ornithine	60.81	0.58 (0.50, 0.66)	74.82	41.94	85.43	26.80	0.15
Proline	356.09	0.55 (0.46, 0.63)	41.13	72.58	87.22	21.33	0.10
Age	9.50	0.39 (0.32, 0.47)	2.13	98.39	85.71	18.10	0.01

neurons, which in turn can cause neurotoxicity. Excessive glutamate levels trigger neuronal dysfunction by causing uncontrolled calcium influx, mitochondrial impairment, and oxidative stress [13]. This can disrupt synaptic pruning and neural network formation, potentially impairing neuronal connectivity and brain circuit development. Such alterations may underlie the characteristic behavioral and cognitive challenges in ASD, with chronic excitotoxicity potentially compromising neuronal survival and promoting neurodevelopmental disruptions [27]. Additionally, aspartic acid influences synaptic plasticity and long-term potentiation (LTP) through the activation of NMDA receptors, which are crucial for the formation of learning and memory [28, 29]. Phenylalanine is a precursor of catecholamine neurotransmitters such as dopamine, norepinephrine, and

epinephrine, which play key roles in mood regulation, attention control, and behavioral responses [30]. Elevated levels of phenylalanine may reflect abnormalities in these neurotransmitter metabolic pathways, leading to behavioral and emotional abnormalities in ASD patients [31, 32]. Studies have found that alterations in phenylalanine metabolism in certain animal models can lead to ASD-like behavioral characteristics, such as increased anxiety and reduced social behavior, suggesting a potential role of catecholamine neurotransmitters in ASD [33]. Leucine and isoleucine, as branched-chain amino acids, regulate cell growth, proliferation, and metabolism by activating the mammalian target of rapamycin (mTOR) signaling pathway. Abnormal activation of the mTOR signaling pathway is closely related to various neurodevelopmental disorders, including ASD [34]. Some studies suggest that excessive activation of the mTOR pathway in ASD models may lead to abnormal neuronal development, thereby affecting brain function and behavior [35].

This study also found that tryptophan and valine were significantly negatively correlated with the severity of ASD. As a precursor of serotonin, reduced levels of tryptophan may lead to insufficient serotonin synthesis, which is an important neurotransmitter for regulating mood and behavior [36]. Abnormalities in serotonin function are closely associated with anxiety, depression, and social impairments commonly seen in ASD patients, so the decline in tryptophan levels may partially explain these behavioral issues [37, 38]. Valine, as a branched-chain amino acid, is involved in the regulation of energy metabolism and immune function. Studies have shown that decreased levels of branched-chain amino acids may be closely related to cognitive dysfunction and neurodevelopmental issues, which may provide a potential physiological explanation for some of the symptoms observed in ASD patients. Valine deficiency may affect brain energy metabolism and immune regulation, thereby exacerbating the severity of neurodevelopmental disorders [7, 39]. The discrepancy between our findings and previous studies reporting elevated valine levels in ASD may be attributed to several key factors. Population heterogeneity, including geographical, genetic, and dietary variations, could significantly influence amino acid metabolism [40, 41]. Methodological differences in sample collection, processing, and analysis techniques might also contribute to these inconsistent results. The broad age range of our participants further introduces complexity in metabolic profile interpretation. These findings further support the complex relationship between ASD and amino acid metabolism.

This study has several strengths. Firstly, with 344 participants covering different genders and age groups, our sample size is larger than many previous studies in this field, enhancing the statistical power of our analyses. Secondly, the study employed multi-level statistical analysis methods, not only assessing the univariate associations between amino acids and ASD severity but also revealing complex non-linear relationships through multivariate logistic regression and non-linear regression analyses, providing profound insights into the biological mechanisms of ASD. Finally, the study constructed a predictive model that integrates multiple variables through Nomogram and ROC curves, which demonstrated high accuracy and practicality in the diagnosis of ASD, validating its potential for clinical application.

However, this study also has some limitations. First, participants were recruited only from a single center in a specific geographic area. Second, as this study is cross-sectional, it cannot establish a causal relationship between amino acid concentrations and ASD severity. Third, the study exclusively included ASD participants and lacked a comparison group of typically developing children, which limits our ability to definitively distinguish the amino acid profiles specific to ASD from normal developmental variations. Fourth, the broad age range of participants (4 months to 12 years) presents a significant methodological challenge. This extensive developmental period encompasses critical stages of neurodevelopment, potentially introducing substantial variability in amino acid metabolism. To address this limitation, future research should employ a longitudinal cohort tracking approach, which would allow for comprehensive monitoring of amino acid metabolic changes across different developmental stages. Such a longitudinal study could provide more nuanced insights into the dynamic metabolic patterns associated with ASD progression, potentially identifying age-specific biomarkers and tracking metabolic variations from early childhood through adolescence.

Based on the limitations of our current study, future research should focus on several key directions. First, longitudinal multi-center studies should be conducted with larger sample sizes, incorporating both ASD and typically developing children to establish robust amino acid profile references and track developmental changes. Second, integrative studies that combine amino acid metabolomics with genetic, neuroimaging, and immunological markers could provide a more comprehensive understanding of ASD pathophysiology. Third, mechanistic research should investigate how amino acid metabolism alterations influence neurodevelopmental processes, potentially revealing causal mechanisms or targeted intervention strategies. These approaches will help advance our understanding of ASD and potentially improve diagnostic and therapeutic approaches.

Conclusion

Our study suggested significant associations between various amino acids and the severity of ASD, particularly the possible positive correlations of aspartic acid, glutamic acid, phenylalanine, and leucine/isoleucine with ASD severity, and the potential negative correlations of tryptophan and valine. These findings have offered new directions for further research into the biological mechanisms of ASD and could have provided potential biomarkers for the diagnosis and treatment of ASD.

Abbreviations

ASD	Autism spectrum disorder
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
RCS	Restricted cubic spline
CARS	Childhood autism rating scale
BCAAs	Branched-chain amino acids
DBS	Dried blood spot
ROC	Receiver operating characteristic
AUC	Area under the curve
DCA	Decision curve analysis

Supplementary Information

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Supplementary Material 1

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Author contributions

J.L&Z.P: Methodology, Writing - Original Draft, Investigation. B.L&W.Y&Y.X: Writing–original draft, Methodology, Investigation. D.W&F.G: Resources, Writing - Review & Editing. Y.Y&L.N: Data Curation, Investigation. L.P&L.Y: Visualization. Z.Q&M.X: Resources, Supervision.

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Data availability

The data used and analysed in this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This project was reviewed by the Ethics Committee of the First Affiliated Hospital of Henan University of Chinese Medicine and complies with the relevant provisions of the Measures for the Ethical Review of Biomedical Research Involving Humans and the Declaration of Helsinki regarding human biological experiments. The study protocol has been approved (Approval No.: 2023HL-427-01), and informed consent was obtained from all primary guardians.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Clinical trial

Not applicable.

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