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Original Article

Association between delayed methotrexate metabolism, coagulation function, and adverse reactions in patients with acute lymphoblastic leukemia receiving high-dose methotrexate treatment

Jing Xu[1], Guoqiang Huang[2], Xiaopeng Liu[2], Xiaoying Zhao[2], Xin Chen[2]

[1]Department of Hematology, Xi'an Gaoxin Hospital, No. 16 Tuanjie South Road, Xi'an High-Tech Zone, Xi'an 710075, Shaanxi, China; [2]Department of Hematology, Hanzhong Central Hospital, No. 557 Middle Section of Laodong West Road, Hantai District, Hanzhong 723000, Shaanxi, China

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Abstract: Objective: This study aimed to identify the risk factors associated with delayed drug metabolism during high-dose methotrexate (HD-MTX) therapy and to analyze the relationship between delayed metabolism and post-treatment toxic adverse effects. Methods: A retrospective analysis was performed on 189 patients with acute lymphoblastic leukemia who received HD-MTX therapy at Xi'an Gaoxin Hospital between February 2018 and May 2023. Serum MTX concentrations were measured at 24, 48, and 72 hours after each HD-MTX administration (cycle), with a 48-hour concentration ≥ 1 μmol/L defining delayed metabolism on a per-cycle basis. Clinical characteristics and laboratory parameters were collected, and univariate and multivariate logistic regression analyses were conducted using SPSS version 27.00 and R version 4.3.3 to determine the risk factors for delayed metabolism. Receiver operating characteristic (ROC) curve analysis was used to evaluate the predictive performance of each significant factor. Results: Significant differences were observed between the delayed cycles (n = 105) and the non-delayed cycles (n = 450) across several clinical and laboratory variables, including age, body mass index (BMI), MTX dosage, activated partial thromboplastin time (APTT), and D-dimer (DD). Logistic regression analysis identified age, BMI, body surface area, MTX dosage, APTT, fibrinogen, DD, albumin, creatinine clearance rate, and phosphorus levels as independent risk factors for delayed metabolism. ROC curve analysis demonstrated that DD exhibited high predictive accuracy for delayed metabolism (area under the curve = 0.833). Moreover, delayed metabolism was significantly associated with a higher incidence of treatment-related toxicities, including mucosal injury, myelosuppression, renal impairment, and gastrointestinal reactions. Conclusion: Delayed MTX metabolism is influenced by multiple clinical and biochemical factors, with DD emerging as a key predictor. Patients experiencing delayed metabolism are at greater risk for severe treatment-related toxicities. Clinicians should closely monitor these high-risk patients and consider timely preventive or corrective interventions to mitigate adverse outcomes.

Keywords: High-dose methotrexate, delayed metabolism, acute lymphoblastic leukemia, risk factors, treatment-related toxicity

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant hematologic disorder characterized by the uncontrolled proliferation of immature lymphoid cells within the bone marrow [1]. Although ALL occurs in both pediatric and adult populations, its incidence is markedly higher in children, whereas adult-onset disease generally exhibits a less favorable prognosis [2]. The clinical manifestations of ALL are heterogeneous and may include anemia, bleeding tendencies, increased susceptibility to infections, and weight loss [3]. With ongoing advancements in therapeutic strategies, treatment outcomes for ALL have improved considerably, particularly owing to the optimization of chemotherapy regimens that have significantly prolonged survival in many patients [4].

Nevertheless, the treatment of ALL continues to encounter substantial challenges, particu-

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larly concerning drug metabolism and excretion when high-dose regimens are administered, as these factors critically influence both therapeutic efficacy and the spectrum of adverse reactions [5]. Methotrexate (MTX), a cornerstone in ALL treatment protocols, plays an indispensable role, especially in high-dose regimens. However, delayed drug metabolism and associated complications - such as coagulation dysfunction and various treatment-related toxicities - remain persistent clinical concerns [6]. MTX acts as an antimetabolite widely used in the management of several diseases, including ALL and rheumatoid arthritis [7]. In the treatment of ALL, MTX is commonly combined with other chemotherapeutic agents to induce tumor cell death by inhibiting DNA synthesis and repair mechanisms. High-dose methotrexate (HD-MTX) regimens have therefore become an integral component of standard therapeutic strategies for ALL [8].

Although MTX demonstrates significant therapeutic efficacy in the treatment of ALL, the inherent instability of its metabolic processes poses substantial clinical challenges. The absorption, distribution, metabolism, and excretion of MTX are influenced by multiple factors and exhibit considerable interindividual variability [9]. Delayed metabolism may lead to excessive plasma drug concentrations, consequently inducing severe toxic reactions [10]. Previous studies have shown that incomplete MTX metabolism or delayed excretion can aggravate treatment-related toxicities, including myelosuppression, hepatic and renal dysfunction, and mucosal injury, thereby diminishing therapeutic efficacy [11].

Drug metabolism refers to the enzymatic transformation of medications into other chemical forms following systemic administration [12]. The metabolism of MTX primarily depends on hepatic and renal function, with renal excretion playing a particularly critical role. When renal insufficiency occurs, MTX clearance is reduced, leading to elevated plasma concentrations that may trigger toxic reactions [13]. Previous studies have demonstrated that patients with renal impairment have a significantly higher risk of delayed MTX metabolism during HD-MTX therapy, which can further aggravate renal injury and myelosuppression [14]. Moreover, delayed MTX metabolism may impair coagulation function, predisposing patients to hemorrhage or thrombotic complications. Elevated systemic MTX levels can disrupt hepatic synthesis of coagulation factors, thereby inducing coagulation abnormalities that increase bleeding risk and exacerbate treatment-related adverse events [15].

Patients undergoing HD-MTX therapy frequently experience treatment-related toxicities, and delayed drug metabolism can further exacerbate these adverse effects by increasing renal burden and potentially inducing acute kidney injury [16]. Therefore, close monitoring of drug metabolism status - particularly renal function and plasma drug concentrations - is essential for the early identification of high-risk patients, enabling timely adjustments to treatment regimens and reducing the incidence and severity of adverse reactions.

This study aims to investigate the associations between delayed drug metabolism following HD-MTX therapy in patients with ALL and alterations in coagulation function and treatment-related adverse reactions. By comprehensively analyzing drug metabolism, coagulation parameters, and adverse events, we seek to identify the key factors influencing MTX metabolism. The findings of this study are expected to provide scientific evidence to support the clinical application of HD-MTX therapy, thereby optimizing therapeutic outcomes while minimizing treatment-related toxicities.

Methods and materials

General information

This retrospective study included 189 unique patients diagnosed with ALL who received HD-MTX therapy at Xi'an Gaoxin Hospital between February 2018 and May 2023. The observation unit was the individual HD-MTX administration (cycle); patients could receive more than one cycle.

Inclusion and exclusion criteria

Inclusion criteria: (1) Patients who met the diagnostic and treatment guidelines for adult ALL in China (2016 edition) [17]. (2) Patients who received one or more cycles of HD-MTX therapy. (3) Patients with relatively complete clinical data.

Exclusion criteria: (1) Patients presenting with severe infections or signs of disseminated intravascular coagulation prior to chemotherapy. (2) Patients who died from severe complications following asparaginase administration or who discontinued treatment prematurely. (3) Patients who received human fibrinogen (FIB), cryoprecipitate, or plasma transfusions before asparaginase administration. (4) Patients with hereditary coagulation factor deficiencies. (5) Patients with incomplete clinical data.

Definition of metabolism delay

During MTX therapy, venous blood samples (2 mL) were collected at 24, 48, and 72 hours after drug administration. Serum MTX concentrations were measured using high-performance liquid chromatography (HPLC). The 48-hour MTX concentration was used to determine delayed metabolism per administration (cycle): (1) Normal excretion (non-delayed group): 48-hour MTX concentration < 1 μmol/L. (2) Delayed excretion (delayed group): 48-hour MTX concentration ≥ 1 μmol/L [18].

Data collection

Clinical data collection: Baseline clinical data were obtained from electronic medical records and outpatient follow-up records. The collected variables included age, sex, body mass index (BMI), disease subtype, chemotherapy regimen, number of treatment cycles, body surface area, and MTX dosage. These parameters were used to evaluate the patient's general physical condition and to provide contextual information regarding treatment background and regimen variations. In particular, body surface area and MTX dosage were essential for analyzing individualized treatment effects. All clinical and laboratory variables used for modeling were recorded per cycle (i.e., within 24 hours before each HD-MTX administration).

Laboratory data collection: Laboratory data were obtained from electronic medical records and outpatient follow-up documentation. All laboratory parameters were measured using peripheral blood samples collected within 24 hours before the initiation of each HD-MTX treatment cycle. The measured indices included activated partial thromboplastin time (APTT), prothrombin time (PT), FIB, D-dimer (DD), albumin (Alb), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), total bilirubin (TBil), creatinine clearance rate (CrCl), calcium (Ca), phosphorus (P), and uric acid. Plasma MTX concentrations were determined using HPLC with fluorescence detection on an Agilent 1260 Infinity system (Agilent Technologies, USA) equipped with a C18 reversed-phase column (4.6 × 150 mm, 5 μm; ZORBAX Eclipse Plus, Agilent). The mobile phase consisted of 0.1 M sodium phosphate buffer (pH 6.0) and acetonitrile (85:15, v/v) at a flow rate of 1.0 mL/min. Analytical reagents and calibration standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Method validation demonstrated excellent precision, with intra- assay and inter-assay coefficients of variation of < 5% and < 8%, respectively.

Toxic side effect records: Treatment-related toxicities were documented using electronic medical records and outpatient follow-up data. The major adverse effects included mucosal injury (e.g., oral ulcers), myelosuppression (e.g., leukopenia, anemia, thrombocytopenia), hepatic dysfunction (e.g., elevated liver enzymes), renal impairment (e.g., increased serum creatinine and decreased glomerular filtration rate), and gastrointestinal toxicities (e.g., nausea, vomiting, gastrointestinal bleeding).

Laboratory testing

All peripheral blood samples were collected within 24 hours before the administration of HD-MTX. Following standard processing procedures, the samples were submitted to the clinical laboratory for analysis.

- Coagulation function testing: APTT, PT, FIB, and DD levels were measured using a Heisenmeyer automated coagulation analyzer. Peripheral blood samples were anticoagulated with sodium citrate, centrifuged to separate plasma, and analyzed automatically.

- Biochemical testing: Alb, Ca, P, and uric acid levels were measured using a Beckman Coulter AU7500 automated biochemical analyzer. Liver and renal function parameters, including AST, ALT, GGT, and TBil, were also assessed with this instrument. CrCl, an indicator of renal function, was calculated using standard formulas.

Assessment time points for toxic side effects

Treatment-related toxicities were monitored at predefined time points following each HD-MTX cycle. Renal and hepatic function parameters, including serum creatinine, CrCl, AST, ALT, and TBil, were evaluated at baseline and within 72 hours after treatment, as related toxicities typically occur early. Mucosal and gastrointestinal events, such as oral ulcers, bleeding, nausea, vomiting, and diarrhea, were assessed on days 3-5, corresponding to their usual onset period. Hematologic toxicity, including leukopenia, anemia, and thrombocytopenia, was evaluated on days 7-14, coinciding with the expected hematologic nadir. These assessment intervals ensured the detection of both early and delayed MTX-related toxicities.

Outcome measurements

Primary outcome: Identification of risk factors associated with delayed MTX metabolism following HD-MTX therapy.

Secondary outcomes: (1) Statistical analysis of the number of patients across different treatment cycles and total MTX dosage. (2) Comparison of clinical characteristics and laboratory parameters between the delayed and non-delayed metabolism groups. (3) Logistic regression analysis was used to evaluate the predictive value of various risk factors for delayed metabolism. (4) Analysis of the relationship between delayed MTX excretion and treatment-related toxicities.

Statistical analysis

All statistical analyses were conducted using SPSS version 27.00 (IBM Corp., Armonk, NY, USA) and R software version 4.3.3 (R Foundation for Statistical Computing, Vienna, Austria). Analyses were performed at the cycle level; "n" denotes HD-MTX administrations (cycles), and a given patient could contribute multiple cycles. Logistic regression modeled the odds of delayed metabolism per cycle. The Kolmogorov-Smirnov test was applied to assess the normality of continuous variable distributions. Categorical variables were analyzed using the chi-square test, while independent-sample t-tests were employed to compare mean differences between groups. For non-normally distributed data, the Mann-Whitney U test was performed using the *wilcox.test()* function in R. Pearson's correlation analysis was conducted to evaluate linear associations between continuous variables via the *cor.test()* function in R. Receiver operating characteristic (ROC) curve analysis was used to assess the discriminative performance of different models, with the *pROC* package and *roc()* function utilized to plot and calculate the area under the curve (AUC). Logistic regression analysis was conducted using the *glm()* function in R to examine the effects of various clinical and laboratory variables on delayed MTX metabolism. Comparisons of AUC values between models were performed using DeLong's test implemented in the *pROC* package (*roc.test()* function). All statistical tests were two-tailed, with a P value < 0.05 being considered statistically significant.

Results

Analysis of the number of patients and total MTX doses across different treatment cycles

The numbers of unique patients and the total HD-MTX administrations (cycles) across treatment rounds were summarized. The results showed that 46 patients received treatment during Cycle 1, accounting for a total of 46 MTX cycles; with 36 patients in Cycle 2 (72 MTX cycles); 36 patients in Cycle 3 (108 MTX cycles); 41 patients in Cycle 4 (164 MTX cycles); 15 patients in Cycle 5 (75 MTX cycles); and 15 patients in Cycle 6 (90 MTX cycles). Across all evaluable administrations, cycles were categorized as delayed (n = 105) or non-delayed (n = 450) based on the 48-hour MTX concentration threshold (**Figure 1**).

Comparison of clinical data between groups

A comparison of baseline clinical characteristics revealed significant differences between the delayed cycles (n = 105) and the non-delayed cycles (n = 450) in terms of age ($P < 0.001$), BMI ($P = 0.006$), number of treatment cycles ($P < 0.001$), body surface area ($P < 0.001$), and MTX dose ($P < 0.001$). However, no significant differences were observed between the two groups with respect to sex ($P = 0.276$), disease subtype ($P = 0.373$), chemotherapy regimen ($P = 0.078$), smoking history ($P = 0.178$), alcohol consumption ($P = 0.194$), diabetes mellitus ($P = 0.236$), or hypertension history ($P = 0.373$) (all $P > 0.05$) (**Table 1**).

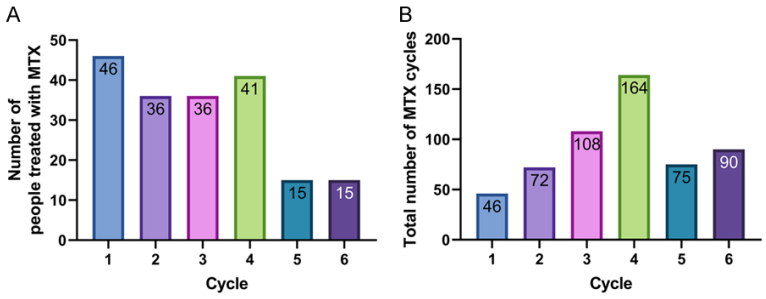

Figure 1. Distribution of patients and total HD-MTX administrations (cycles) across treatment rounds. A. Number of unique patients who received HD-MTX in each round. B. Total number of HD-MTX administrations (cycles) across rounds. Note: Subsequent efficacy/toxicity analyses are performed at the cycle level (n denotes cycles). MTX, methotrexate.

Comparison of laboratory indices before treatment

A comparison of baseline laboratory parameters revealed significant differences between the delayed cycles (n = 105) and the non-delayed cycles (n = 450) in APTT ($P < 0.001$), PT ($P < 0.001$), FIB ($P < 0.001$), DD ($P < 0.001$), Alb ($P < 0.001$), CrCl ($P < 0.001$), and P ($P < 0.001$). However, no significant differences were observed in AST ($P = 0.785$), ALT ($P = 0.697$), GGT ($P = 0.791$), TBil ($P = 0.082$), Ca ($P = 0.192$), or uric acid ($P = 0.130$) (all $P > 0.05$) (**Table 2**).

Correlation analysis of differential indicators

Correlation analysis was conducted for variables that showed significant differences between delayed and non-delayed cycles. Significant correlations ($P < 0.05$) were observed among the following parameters: APTT with PT ($r = 0.055$), APTT with FIB ($r = 0.09$), APTT with DD ($r = -0.166$), APTT with Alb ($r = 0.096$), APTT with CrCl ($r = 0.113$), PT with FIB ($r = 0.143$), FIB with CrCl ($r = 0.086$), DD with Alb ($r = -0.142$), DD with CrCl ($r = -0.236$), and CrCl with P ($r = 0.102$). All correlation coefficients (r values) were less than 0.3, indicating weak associations with minimal collinearity risk. Therefore, all differential indicators were retained for subsequent regression analysis (**Figure 2**).

Classification transformation and logistic regression variable assignment for differential quantitative indicators

For logistic regression analysis, the differential quantitative indicators were classified according to cut-off values determined by ROC curve analysis (Figure S1). Among these indicators, DD exhibited the highest AUC value of 0.833 (95% confidence interval: 0.791-0.875), with a specificity of 73.11%, sensitivity of 80.00%, and a Youden's index of 53.11%. The optimal cut-off value for DD was 1.185, indicating strong discriminative ability for identifying delayed metabolism. In contrast, PT showed the lowest AUC value of 0.607 (95% confidence interval: 0.546-0.668), with a specificity of 78.89%, sensitivity of 39.05%, and a Youden's index of 17.94%. The corresponding cut-off value for PT was 11.5, reflecting relatively poor discriminative performance (**Table 3**). Subsequently, the quantitative variables were classified based on the respective cut-off values and assigned categorical values for logistic regression analysis (**Table 4**).

Logistic regression analysis of factors associated with delayed methotrexate metabolism

Univariate and multivariate logistic regression analyses were conducted at the cycle level to identify factors associated with delayed MTX metabolism per administration. The univariate logistic regression analysis demonstrated that age (odds ratio [OR] = 2.635, $P < 0.001$), BMI (OR = 1.915, $P = 0.007$), body surface area (OR = 0.221, $P < 0.001$), MTX dose (OR = 0.225, $P < 0.001$), APTT (OR = 0.205, $P < 0.001$), PT (OR = 0.418, $P < 0.001$), FIB (OR = 0.294, $P < 0.001$), DD (OR = 10.876, $P < 0.001$), Alb (OR = 0.133, $P < 0.001$), CrCl (OR = 0.228, $P < 0.001$), and P (OR = 0.443, $P < 0.001$) were all significantly correlated with delayed MTX metabolism. In contrast, the number of treatment cycles was not statistically significant (OR = 1.301, $P = 0.090$) (**Figure 3**). In the multivariate logistic regression analysis, age (OR = 2.519, $P = 0.021$), BMI (OR = 5.856, $P < 0.001$), body surface area (OR = 0.312, $P = 0.004$), MTX dose (OR = 0.107, $P < 0.001$), APTT (OR = 0.100, $P < 0.001$), FIB (OR = 0.131, $P < 0.001$), DD (OR = 10.694, $P < 0.001$), Alb (OR = 0.075, $P < 0.001$), CrCl (OR = 0.261, $P < 0.001$), and P (OR = 0.269, $P < 0.001$) remained significant independent predictors of delayed MTX metab-

Table 1. Comparison of clinical characteristics between delayed and non-delayed cycles

Variable	Total	Delayed Group (n = 105)	non-delayed cycles (n = 450)	Statistic Value	*P*-value
Age (years)	64.00 [61.50, 67.50]	66.00 [64.00, 70.00]	64.00 [61.00, 67.00]	4.604	< 0.001
Sex					
Male	328	67	261	1.189	0.276
Female	227	38	189		
BMI (kg/m^2)					
≥ 25	124	34	90	7.521	0.006
< 25	431	71	360		
Disease subtype					
B-ALL	448	88	360	0.794	0.373
T-ALL	107	17	90		
Chemotherapy regimen					
Single HD-MTX	484	97	387	3.107	0.078
Combined with other agents	71	8	63		
Number of treatment cycles					
≤ 2	118	25	93	14.395	< 0.001
3-4	272	35	237		
≥ 5	165	45	120		
Body surface area (m^2)	1.06±0.37	0.89±0.29	1.10±0.37	5.415	< 0.001
MTX dose	4.52±1.92	3.47±1.63	4.77±1.90	6.472	< 0.001
Smoking history					
Yes	354	61	293	1.814	0.178
No	201	44	157		
Alcohol consumption					
Yes	112	26	86	1.688	0.194
No	443	79	364		
Diabetes mellitus					
Yes	90	13	77	1.402	0.236
No	465	92	373		
Hypertension History					
Yes	157	26	131	0.794	0.373
No	398	79	319		

Note: MTX, methotrexate; BMI, body mass index; HD-MTX, high-dose methotrexate; B-ALL, B-cell acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia.

olism. However, the number of treatment cycles and PT were not statistically significant in the multivariate model (both P > 0.05) (**Figure 4**).

ROC curve analysis of logistic regression variables for predicting delayed MTX metabolism

ROC curve analysis was conducted at the cycle level to evaluate the predictive performance of ten differential variables for delayed MTX metabolism. **Figure 5A** illustrates the ROC curves of ten differential variables, while **Figure 5B** presents the comparison matrix of the corresponding AUCs. The analysis revealed that DD had the highest AUC value, indicating superior accuracy in predicting delayed MTX metabolism. Although the remaining variables demonstrated relatively lower AUCs, they still exhibited certain predictive value. The AUC comparison matrix further showed statistically significant differences between specific variables (P < 0.05), suggesting heterogeneity in their predictive performance (**Figure 5**).

Relationship between delayed MTX excretion and post-treatment toxic side effects

The association between delayed MTX excretion and post-treatment toxic side effects was analyzed at the cycle level (denominators refer to cycles). Significant differences were observed between the delayed and non-delayed groups

Table 2. Comparison of laboratory parameters between delayed and non-delayed cycles

Index	Total	Delayed Group (n = 105)	non-delayed cycles (n = 450)	Statistic Value	*P*-value
APTT (s)	35.46 [33.36, 37.88]	33.67 [30.86, 35.24]	35.95 [33.79, 38.31]	7.473	< 0.001
PT (s)	12.94±2.03	12.25±2.10	13.10±1.98	3.926	< 0.001
FIB (g/L)	3.75±1.18	3.18±1.04	3.88±1.18	5.591	< 0.001
DD (μg/mL)	1.01 [0.64, 1.36]	1.52 [1.23, 1.88]	0.90 [0.55, 1.22]	10.639	< 0.001
Alb (g/dL)	3.56±0.18	3.41±0.19	3.60±0.17	10.146	< 0.001
AST (U/L)	19.90±2.99	19.83±3.22	19.92±2.93	0.273	0.785
ALT (U/L)	20.34±4.63	20.18±4.51	20.38±4.66	0.390	0.697
GGT (U/L)	40.54±10.08	40.77±13.36	40.48±9.17	-0.265	0.791
TBil (mg/dL)	0.40±0.08	0.39±0.08	0.40±0.08	1.741	0.082
CrCl (mL/min)	82.53±10.92	75.89±9.73	84.08±10.60	7.233	< 0.001
Ca (mg/dL)	8.66±0.47	8.61±0.41	8.68±0.49	1.307	0.192
P (mg/dL)	3.26±0.38	3.14±0.37	3.29±0.38	3.664	< 0.001
UA (mg/dL)	4.32±0.47	4.38±0.57	4.31±0.45	-1.515	0.130

Note: APTT, activated partial thromboplastin time; PT, prothrombin time; FIB, fibrinogen; DD, D-dimer; Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; TBil, total bilirubin; CrCl, creatinine clearance rate; Ca, calcium; P, phosphorus; UA, uric acid.

in the incidence of mucosal injury, bone marrow suppression, renal dysfunction, and gastrointestinal adverse effects (all $P < 0.05$). Specifically, the delayed group exhibited markedly higher incidences of mucosal injury ($P < 0.001$), bone marrow suppression ($P < 0.001$), renal dysfunction ($P = 0.005$), and gastrointestinal side effects ($P < 0.001$) compared with the non-delayed group. However, no significant difference was detected in the occurrence of liver dysfunction between the two groups ($P = 0.609$) (**Table 5**).

Discussion

HD-MTX is a cornerstone of ALL therapy, exerting potent cytotoxic effects on rapidly proliferating tumor cells by inhibiting dihydrofolate reductase and thereby blocking DNA synthesis [19]. However, the pharmacokinetics of HD-MTX exhibit substantial interindividual variability. In certain patients, delayed MTX metabolism may occur, leading to persistently elevated plasma concentrations and a consequent increase in the risk of severe toxic reactions [20]. In this retrospective study involving 189 patients with ALL who received HD-MTX therapy, we systematically analyzed plasma MTX concentrations and relevant clinical parameters to elucidate the determinants of delayed MTX metabolism and its associations with coagulation function and treatment-related toxicity. Our findings indicate that factors such as age, BMI, body surface area, MTX dosage, APTT, FIB, DD, Alb, CrCl, and P levels may influence MTX metabolism to varying degrees. Because HD-MTX dosing, monitoring, and leucovorin rescue are managed per administration, our analyses were performed at the cycle level; therefore, the numbers reported for delayed (n = 105) and non-delayed (n = 450) refer to administrations (cycles) rather than unique patients.

First, elderly patients and those with higher BMI demonstrated a higher risk of delayed MTX metabolism. In our study, older patients frequently exhibited diminished hepatic and renal function, which may impair drug clearance even when routine renal function indicators remain within the normal range [21, 22]. Ikeda et al. reported that advanced age and repeated cycles of HD-MTX therapy were significant predictors of delayed MTX clearance in adult patients [18]. This finding is consistent with our results, indicating that age-related physiological decline contributes to reduced elimination capacity and a higher likelihood of metabolism delay. Furthermore, patients with elevated BMI may experience altered pharmacokinetic distribution and an extended circulation half-life due to increased adipose tissue, resulting in prolonged MTX retention. This mechanism aligns with the findings of Misaka et al., who observed that higher BMI was associated with delayed MTX clearance during HD-MTX treatment [23].

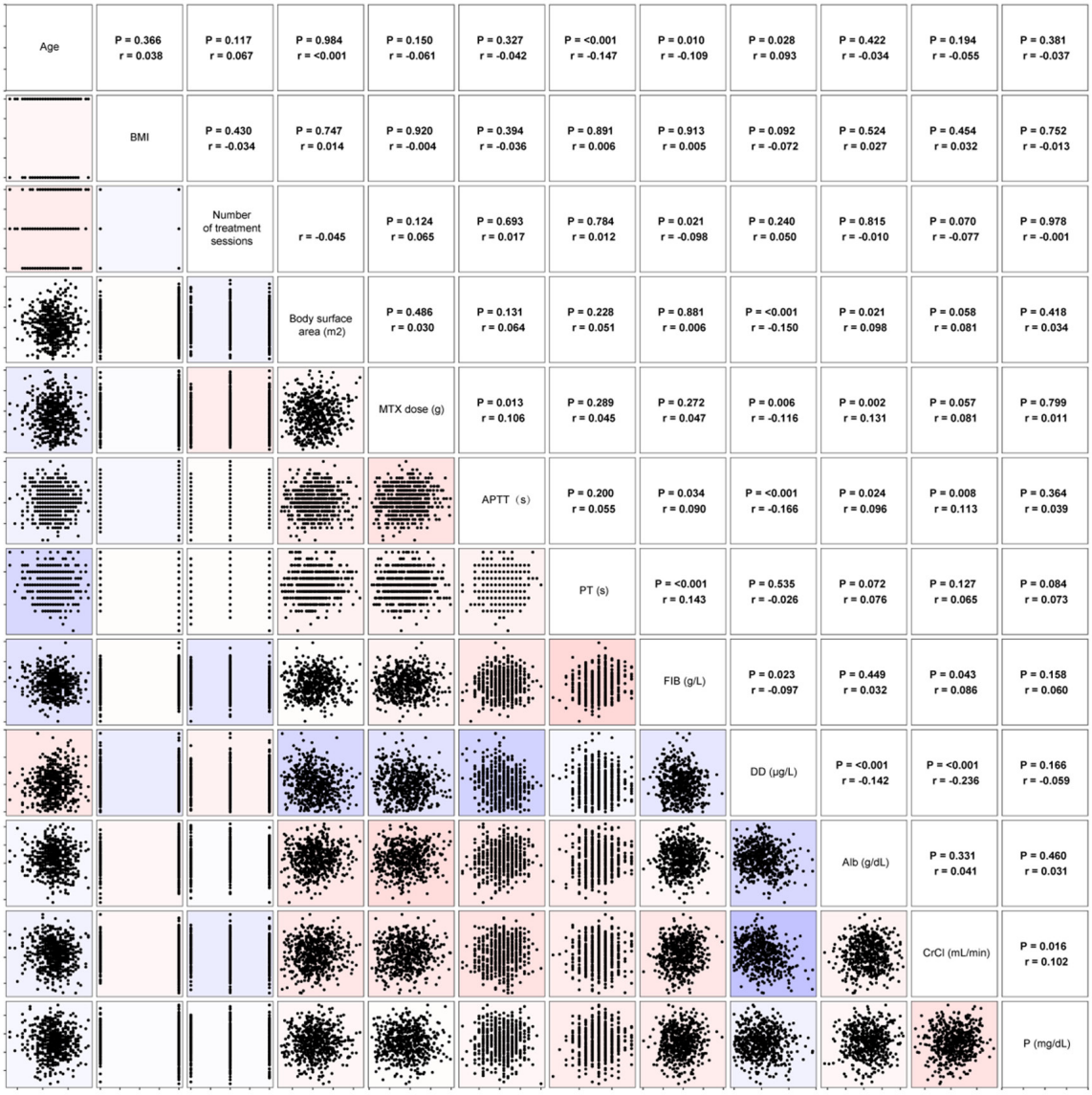

Figure 2. Correlation analysis of differential indicators. Note: BMI, body mass index; MTX, methotrexate; APTT, activated partial thromboplastin time; PT, prothrombin time; FIB, fibrinogen; DD, D-dimer; Alb, albumin; CrCl, creatinine clearance rate; P, phosphorus.

Regarding laboratory indices, we observed that prolonged APTT was negatively correlated with delayed MTX metabolism, suggesting a potential link between coagulation function and MTX clearance. Yu et al. reported that elevated DD levels were strongly associated with delayed MTX metabolism, with DD demonstrating a high predictive accuracy (AUC = 0.833) for identifying patients at risk [24]. Although the underlying mechanisms remain incompletely understood, several hypotheses may explain this relationship. One possibility is that MTX exposure affects the coagulation-fibrinolysis balance through endothelial injury, which in turn alters fibrin turnover. Nevertheless, our findings represent associative rather than causal relationships. The observed correlation between DD levels and delayed MTX clearance may reflect shared pathophysiological mechanisms or confounding clinical factors not fully accounted for in this analysis. Therefore, while DD appears to serve as a promising predictive biomarker for delayed MTX metabolism, further mechanistic studies are warranted to clarify the causal interplay between coagulation activation and MTX pharmacokinetics. With respect

Table 3. Cut-off values for differential quantitative indicators determined by ROC curve analysis

Marker	AUC	95% CI	Specificity (%)	Sensitivity (%)	Youden's Index (%)	Cut-off Value
Age (years)	0.644	0.588-0.699	46.44%	75.24%	21.68%	63.5
Body surface area (m^2)	0.678	0.628-0.729	53.11%	80.00%	33.11%	1.09
MTX dose (g)	0.698	0.646-0.751	62.89%	72.38%	35.27%	4.135
APTT (s)	0.73	0.681-0.779	56.44%	79.05%	35.49%	35.5
PT (s)	0.607	0.546-0.668	78.89%	39.05%	17.94%	11.5
FIB (g/L)	0.675	0.621-0.729	56.44%	72.38%	28.83%	3.745
DD (μg/mL)	0.833	0.791-0.875	73.11%	80.00%	53.11%	1.185
Alb (g/dL)	0.773	0.719-0.828	78.22%	67.62%	45.84%	3.475
CrCl (mL/min)	0.713	0.661-0.764	67.78%	67.62%	35.40%	79.195
P (mg/dL)	0.609	0.549-0.669	63.78%	56.19%	19.97%	3.175

Note: APTT, activated partial thromboplastin time; PT, prothrombin time; FIB, fibrinogen; DD, D-dimer; Alb, albumin; CrCl, creatinine clearance rate; P, phosphorus; MTX, methotrexate; ROC, receiver operating characteristic; AUC, area under the curve; CI, confidence interval.

Table 4. Variable classification and assignment for logistic regression analysis

Variable	Variable Type	Assignment Criteria
Age	(X)	< 63.5 = 0, ≥ 63.5 = 1
BMI (kg/m^2)	(X)	< 25 kg/m^2 = 0, ≥ 25 kg/m^2 = 1
Number of treatment cycles	(X)	≤ 2 = 0, 3-4 = 1, ≥ 5 = 2
Body surface area (m^2)	(X)	< 1.09 = 0, ≥ 1.09 = 1
MTX dose (g)	(X)	< 4.135 = 0, ≥ 4.135 = 1
APTT (s)	(X)	< 35.5 = 0, ≥ 35.5 = 1
PT (s)	(X)	< 11.5 = 0, ≥ 11.5 = 1
FIB (g/L)	(X)	< 3.745 = 0, ≥ 3.745 = 1
DD (μg/mL)	(X)	< 1.185 = 0, ≥ 1.185 = 1
Alb (g/dL)	(X)	< 3.475 = 0, ≥ 3.475 = 1
CrCl (mL/min)	(X)	< 79.195 = 0, ≥ 79.195 = 1
P (mg/dL)	(X)	< 3.175 = 0, ≥ 3.175 = 1
Delayed metabolism	(Y)	No = 0, Yes = 1

Note: BMI, body mass index; MTX, methotrexate; APTT, activated partial thromboplastin time; PT, prothrombin time; FIB, fibrinogen; DD, D-dimer; Alb, albumin; CrCl, creatinine clearance rate; P, phosphorus.

to hepatic and renal function markers, decreased serum Alb levels and reduced CrCl were significant risk factors for delayed MTX metabolism, consistent with previous studies by Ikeda et al. [18] and Yu et al. [24]. Ikeda's findings indicated that hypoalbuminemia and impaired renal function lead to sustained MTX accumulation, thereby increasing risk of treatment-related toxicity [18, 24]. In our cohort, lower Alb levels likely elevated the unbound fraction of MTX, prolonging its plasma retention, whereas diminished CrCl further reduced renal elimination, resulting in higher systemic exposure and an increased risk of adverse effects [25, 26]. Alterations in serum P levels were also identified as potential contributors to MTX metabolism delay. MTX-induced cytotoxicity can cause cellular lysis and the release of intracellular phosphate, leading to hyperphosphatemia, which may impair renal tubular function and promote calcium-phosphate precipitation, further exacerbating renal injury and reducing MTX clearance. Conversely, hypophosphatemia has been reported during chemotherapy in some patients, possibly due to altered bone metabolism or proximal tubular dysfunction. Both hyperphosphatemia and hypophosphatemia thus reflect MTX-associated disturbances in phosphate homeostasis and may serve as indicators of impaired drug elimination and increased toxicity risk [27]. This mechanistic link supports our observation that abnormal P levels are associated with delayed MTX metabolism.

Clinically, delayed MTX metabolism markedly increases the incidence of treatment-related toxicities, particularly mucosal injury, bone marrow suppression, renal dysfunction, and gastrointestinal adverse effects [28]. Mosleh et al. further demonstrated that delayed MTX

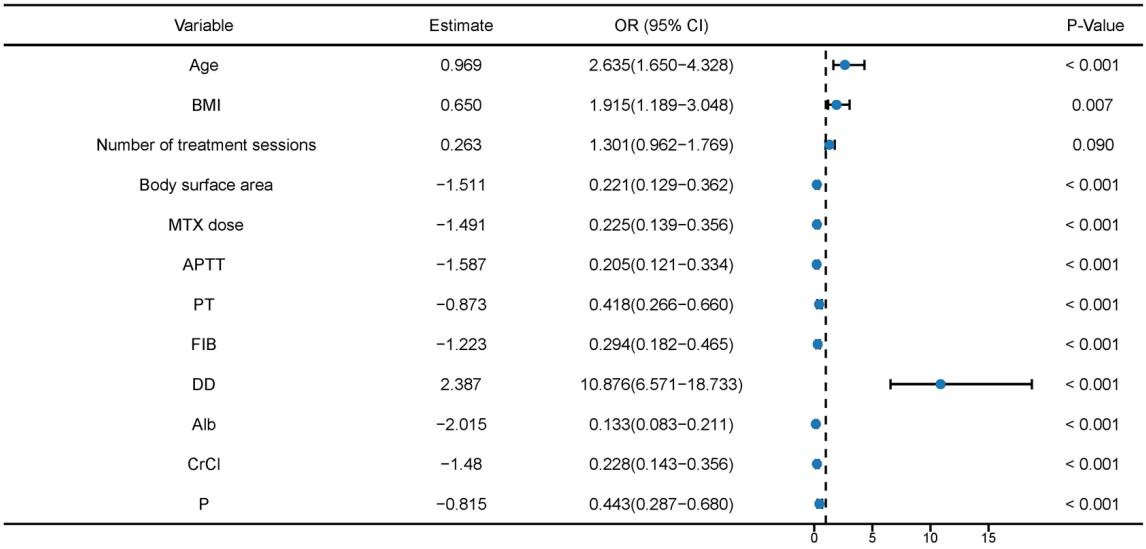

Figure 3. Univariate logistic regression analysis (cycle level) of factors associated with delayed MTX metabolism. Note: BMI, body mass index; MTX, methotrexate; APTT, activated partial thromboplastin time; PT, prothrombin time; FIB, fibrinogen; DD, D-dimer; Alb, albumin; CrCl, creatinine clearance rate; P, phosphorus; OR, odds ratio; CI, confidence interval.

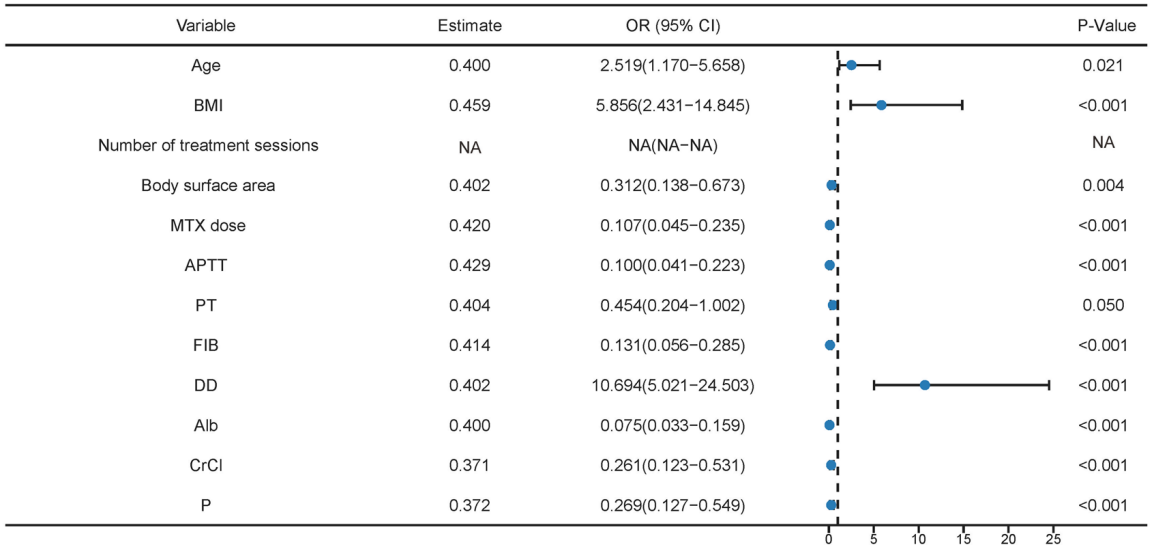

Figure 4. Multivariate logistic regression analysis (cycle level) of factors associated with delayed MTX metabolism. Note: BMI, body mass index; MTX, methotrexate; APTT, activated partial thromboplastin time; PT, prothrombin time; FIB, fibrinogen; DD, D-dimer; Alb, albumin; CrCl, creatinine clearance rate; P, phosphorus; OR, odds ratio; CI, confidence interval.

clearance not only heightens the risk of toxicity but also adversely affects overall clinical outcomes. In particular, renal dysfunction may precipitate a vicious cycle in which impaired MTX elimination exacerbates renal injury, further prolonging drug exposure and worsening clinical manifestations [29]. These findings underscore the importance of early identification of patients at risk for delayed MTX metabolism and the implementation of timely interventions - such as adequate hydration, urine alkalization, and prompt leucovorin rescue - to mitigate toxicity, enhance drug clearance, and ultimately improve therapeutic outcomes.

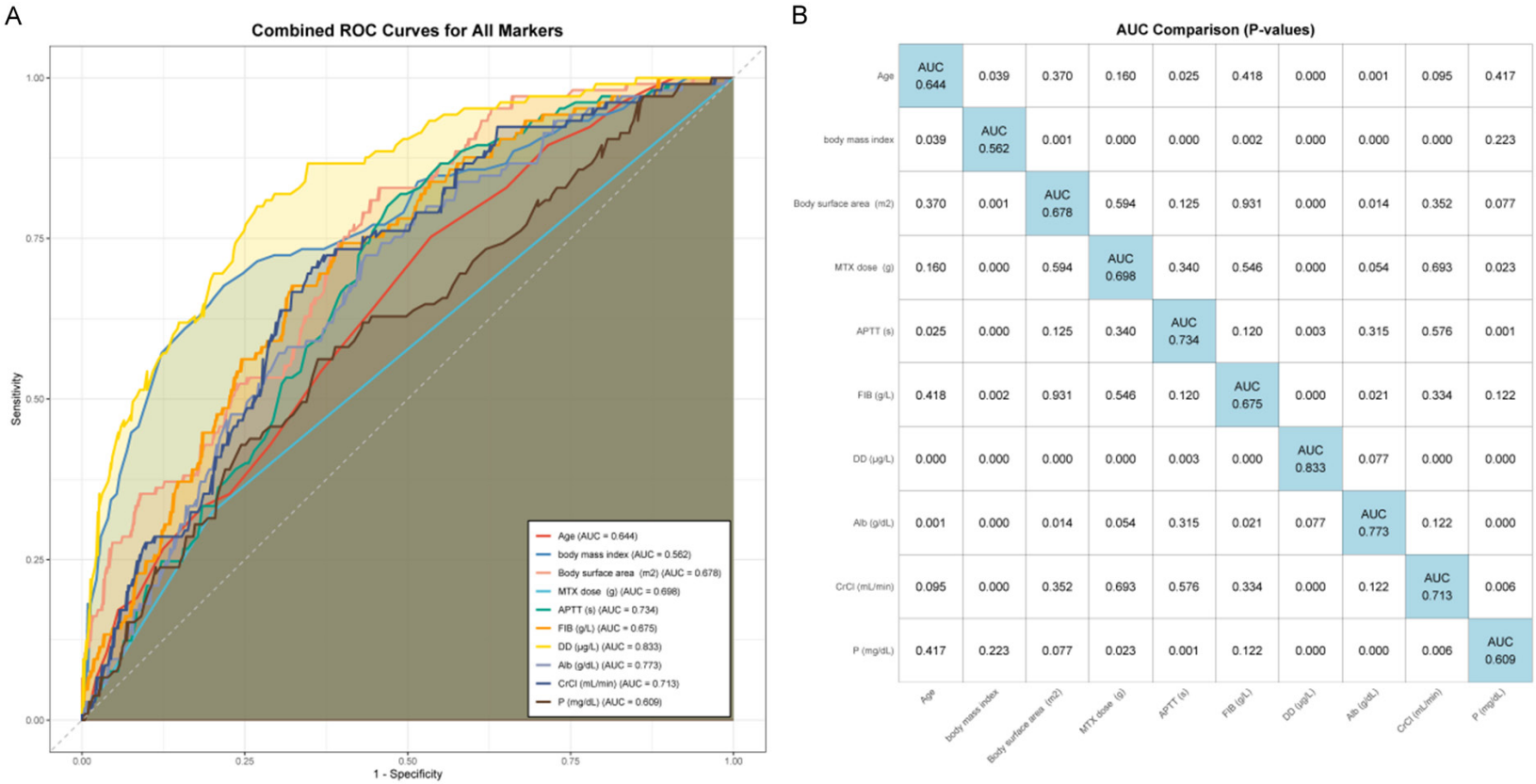

Figure 5. Combined ROC curves (cycle level) of ten differential variables for predicting delayed MTX metabolism. A. Combined ROC curves illustrating the predictive performance of ten differential variables for delayed MTX metabolism. Each curve represents one variable, with the area under the curve (AUC) shown in the legend. B. Comparison matrix of AUC values among the ten variables. Pairwise *P*-values indicate the statistical significance of differences in predictive performance. D-dimer (DD) demonstrated the highest AUC (0.833), indicating superior accuracy in predicting delayed MTX metabolism. Note: BMI, body mass index; MTX, methotrexate; APTT, activated partial thromboplastin time; FIB, fibrinogen; DD, D-dimer; Alb, albumin; CrCl, creatinine clearance rate; P, phosphorus; ROC, receiver operating characteristic.

Table 5. Association between delayed MTX excretion and post-treatment toxic side effects at the cycle level

Group	Mucosal Injury	Bone Marrow Suppression	Liver Dysfunction	Renal Dysfunction	Gastrointestinal Adverse Effects
Delayed group (n = 105)	65/40	32/73	89/16	97/8	40/65
non-delayed cycles (n = 450)	385/65	250/200	390/60	440/10	335/115
Statistic value	31.044	21.425	0.261	7.902	51.331
P-value	< 0.001	< 0.001	0.609	0.005	< 0.001

Note: MTX, methotrexate.

Lian et al. utilized an ultra-high-performance liquid chromatography-tandem mass spectrometry method to monitor MTX concentrations during HD-MTX therapy, highlighting the critical role of precise therapeutic drug monitoring in achieving personalized treatment, minimizing severe adverse effects, and optimizing therapeutic efficacy [30]. In our study, plasma MTX concentrations were monitored using HPLC, providing a reliable and scientific basis for individualized treatment of HD-MTX management. From a technological perspective, the machine learning-based web prediction tool proposed by Jian et al. represents an innovative approach for predicting delayed MTX metabolism in pediatric ALL patients undergoing HD-MTX therapy [31]. By integrating multiple clinical and laboratory parameters, this predictive model assists clinicians in identifying high-risk patients and implementing timely preventive measures to reduce the likelihood of delayed MTX elimination. Such advances in predictive modeling underscore the potential of precision medicine approaches to improve safety and efficacy in HD-MTX-based chemotherapy.

Although this study identified several risk factors associated with delayed MTX metabolism, several limitations should be acknowledged. First, as a retrospective single-center study, the sample size was relatively limited, and both selection bias and information bias could not be completely avoided. Second, although common confounding factors were adjusted for, certain potential influences - such as genetic variability and concomitant medications - were not fully incorporated into the analysis. In particular, polymorphisms in MTX metabolism-related genes (e.g., methylenetetrahydrofolate reductase, solute carrier organic anion transporter family member 1B1) have been recognized as significant determinants of delayed clearance; however, these genetic data were unavailable for many patients in this cohort and were thus excluded to maintain data integrity and minimize bias. Third, despite the standardized MTX administration protocols implemented in our institution - including dose adjustment strategies, hydration, and urine alkalinization - minor variations in clinical practice across patients could not be entirely eliminated, which may have affected the incidence of metabolism delay. Finally, the definition of delayed metabolism in this study was based on a 48-hour plasma MTX concentration ≥ 1 μmol/L. While this criterion is widely accepted, it may not fully reflect the pharmacokinetic heterogeneity among all patients. Future investigations should therefore include multicenter, large-sample prospective studies integrating pharmacogenomic data and multidimensional clinical information to better elucidate the mechanisms underlying MTX metabolism delay and to establish more accurate, individualized risk prediction models.

Conclusion

This study demonstrated that factors such as patient age, BMI, renal function, serum Alb levels, and DD levels related to coagulation function are significantly associated with delayed MTX metabolism. Delayed metabolism markedly increases the risk of treatment-related toxicities. Clinically, enhanced monitoring and timely intervention in high-risk patients - through adequate hydration, urine alkalinization, individualized dose adjustment, and optimized leucovorin rescue - are essential to improve the safety and therapeutic efficacy of HD-MTX therapy and ultimately enhance the prognosis of patients with ALL.

Disclosure of conflict of interest

None.

Address correspondence to: Xin Chen, Department of Hematology, Hanzhong Central Hospital, No. 557 Middle Section of Laodong West Road, Hantai District, Hanzhong 723000, Shaanxi, China. E-mail: seaman099@126.com

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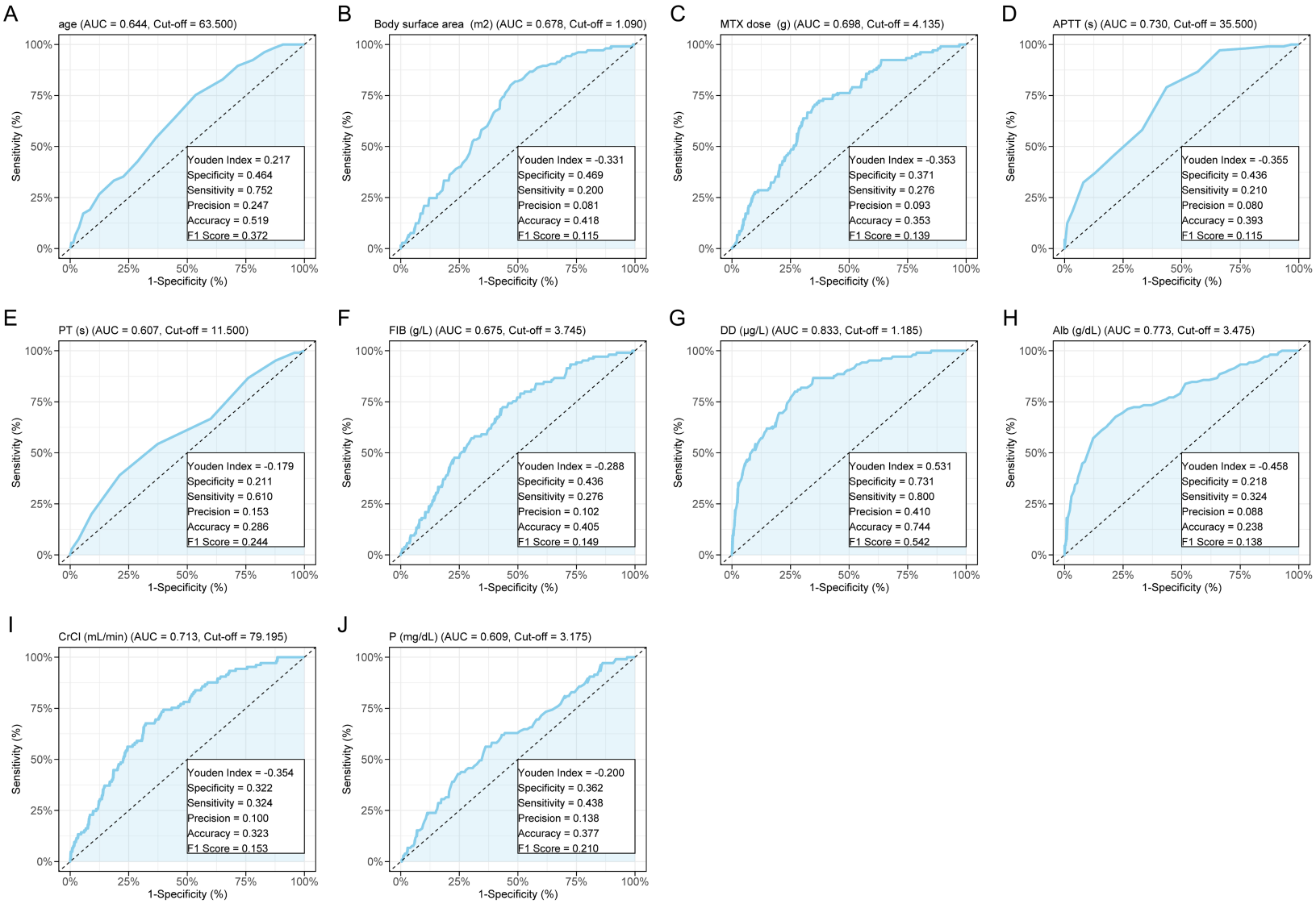

Figure S1. ROC curve confirmation of clinical data and laboratory index measurement data ROC. A. ROC curve of age. B. ROC curve of BMI. C. ROC curve of MTX dose. D. The ROC curves of the APTT. E. The ROC curves of the PT. F. The ROC curve of the FIB. G. The ROC curves of the DD. H. The ROC curves of the Alb. I. The ROC curve of the CrCl. J. The ROC curve of the P. Note: Body mass index (BMI), methotrexate (MTX), activated partial thromboplastin time (APTT), prothrombin time (PT), fibrinogen (FIB), D-dimer (DD), albumin (Alb), creatinine.