LC-MS/MS-based clinical value exploration of the methionine cycle in luminal breast cancer

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Abstract. A group of patients with luminal breast cancer progresses to advanced stages because of endocrine therapy failure. While palbociclib improves the disease prognosis in the clinic, resistance after treatment remains a prominent issue. As a core systemic metabolic component, the methionine cycle currently lacks systematic research on luminal breast cancer and palbociclib resistance. The present study enrolled 146 patients with luminal breast cancer and 36 HCs from Jiangsu Cancer Hospital (Nanjing, China). Liquid chromatography-tandem mass spectrometry was used to detect the methionine cycle-related metabolites in the plasma. The Mann-Whitney U test was used to compare intergroup differences and diagnostic efficacy was evaluated using receiver operating characteristic curve analysis. Least absolute shrinkage and selection operator was applied to screen resistance-related variables, after which a nomogram prediction model was constructed. Plasma levels of methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and homocysteine were lower in patients with luminal breast cancer when compared with HCs, with an AUC of 0.8130 for the combined metabolites in diagnosis. Late-stage patients had lower methionine, SAM, SAH, as well as the SAM/SAH ratio than early-stage patients, with combined metabolites achieving an AUC of 0.9228. Additionally, palbociclib-resistant patients had lower SAM, SAH and homocysteine levels, with combined metabolites showing an AUC of 0.9229 for resistance identification. Finally, five variables were screened and used to construct a nomogram prediction model, with an AUC >0.9000 in the training set and 0.8000 in the internal validation sets. The present results suggest that methionine cycle-related metabolites are promising potential biomarkers for the diagnosis and progression assessment of luminal breast cancer. Furthermore, the constructed nomogram prediction model provides a new strategy for timely clinical treatment and intervention.

Introduction

Breast cancer, the most common malignant tumor among women worldwide, accounts for >2.3 million new cases annually (1). Breast cancer usually arises from the combined effects of multiple factors, including genetic factors, hormone levels, lifestyle and environmental factors, which typically lead to the malignant transformation of breast epithelial cells (2). Among all types of breast cancer, the luminal subtype contributes to ~70% of cases, including luminal A (ERα+, PR+, HER2- and low Ki-67) and luminal B (ERα+, PR+/-, HER2-/+ and high Ki-67) (3). The luminal subtype is a hormone-dependent molecular subtype of breast cancer, and patients at the early stage typically achieve favorable therapeutic outcomes with endocrine therapy. Of all patients, ~20% of patients with the luminal subtype still experience recurrence and distant metastasis because of endocrine therapy resistance, which progresses to advanced-stage disease and results in poor disease prognosis (4,5). Therefore,

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close attention to the dynamic progression of luminal breast cancer is highly important.

The application of CDK4/6 inhibitors has reshaped the therapeutic landscape for patients with endocrine therapy failure. As a first-line clinical CDK4/6 inhibitor, palbociclib has been shown to achieve favorable clinical efficacy and considerably prolong the survival of patients with advanced luminal breast cancer (6). However, the issue of palbociclib resistance has become increasingly prominent, further hindering its widespread clinical application (7). Currently, the clinical determination of therapeutic resistance relies mainly on disease progression-driven tumor protein markers and imaging examinations. However, these approaches exhibit poor specificity and sensitivity in minimal residual disease detection, along with drawbacks such as an inherent time lag (8). Thus, developing innovative efficacy assessment methods and resistance prediction strategies is key for improving patient outcomes.

Tumor initiation and progression are accompanied by metabolic reprogramming, and metabolomics is favorable for identifying biological phenotypes. The methionine cycle, a key component of one-carbon metabolism, is a universal cellular metabolic process that primarily supplies methyl groups for intracellular biological reactions (9). A total of four metabolites, methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and homocysteine, are collectively involved in the methionine cycle. Dysregulation of the methionine cycle usually leads to hypo- or hypermethylation, thereby causing physiological abnormalities and even tumorigenesis (10). Previous studies have shown that the methionine cycle drives tumorigenesis through multiple mechanisms, such as epigenetic regulation (methylation), immune regulation and redox balance (11-13). Previous studies have reported aberrations in the abundance of the methionine cycle-related metabolites in breast cancer tissue compared with normal tissue (14,15). Moreover, SAM can inhibit breast cancer cell proliferation by regulating microRNAs (miRNAs), apoptosis and autophagy (16,17). These findings indicate an intricate and association between the methionine cycle and breast cancer. Nevertheless, the association of the methionine cycle with luminal breast cancer, along with its function and value in tumor progression and palbociclib resistance, still requires further exploration.

In the present study, targeted metabolomics based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) was applied to measure methionine cycle levels in plasma from luminal breast cancer patients and healthy controls (HCs). Intergroup differences in metabolite levels were also analyzed and variations in metabolite abundance across early to advanced disease stages was investigated. In addition, the potential value of the methionine cycle in luminal breast cancer and developed a palbociclib resistance prediction model is clarified. Consequently, the present study sheds light on the metabolic features of luminal breast cancer and provides a new approach for accurate clinical efficacy evaluation and optimization of drug resistance treatment strategies.

Materials and methods

Reagents and chemicals. Standards of the methionine cycle-related metabolites (methionine, SAM, SAH and homocysteine) were obtained from Shanghai Macklin Biochemical Co., Ltd. The isotopic internal standard including Methionine-d3 (Met-d3), S-Adenosylmethionine-d3 (SAM-d3), S-Adenosylhomocysteine-d4 (SAH-d4), and Homocysteine-d4 (Hcy-d4) were purchased from MedChemExpress. LC-MS grade methyl alcohol and acetonitrile were obtained from MilliporeSigma. Ammonium formate and LC-MS grade formic acid were both obtained from Thermo Fisher Scientific, Inc.

Study cohort and blood sample collection. A total of 146 patients with luminal breast cancer and 36 HCs were recruited for plasma sample collection at Jiangsu Cancer Hospital (Nanjing, China) between August 2023 and March 2024, including 94 (64.4%) patients with luminal A subtype and 52 (35.6%) patients with luminal B subtype. The inclusion criteria were as follows: i) Age >18 years; ii) histopathologically confirmed luminal A or luminal B breast cancer based on surgically resected or biopsy tissues; iii) no history of other concurrent malignant tumors or previous malignancies; and iv) patients with locally advanced or advanced luminal breast cancer who subsequently received first-line palbociclib-based therapy, as these patients were further included in the predictive model analysis for palbociclib treatment response. Breast cancer staging was performed according to established clinical staging criteria (18) and molecular subtypes were determined by professional pathologists based on molecular marker expression. Individuals that underwent physical examinations during the same period that had no clinical symptoms or abnormal findings upon examination were recruited as the HCs group. The present study was approved by the Ethics Committee of Jiangsu Cancer Hospital (Nanjing, China) and written informed consent was obtained from all participants.

For patients included in the palbociclib response prediction cohort, peripheral blood samples were collected prior to initiation of palbociclib-based therapy, at the time when patients were confirmed to receive treatment. Patients were subsequently followed longitudinally and classified according to clinical outcomes. Treatment response was evaluated based on the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. Progressive disease was defined as ≥20% increase in the sum of the diameters of target lesions relative to the nadir, with an absolute increase of at least 5 mm or the appearance of new lesions. Palbociclib resistance was operationally defined as RECIST v1.1-confirmed disease progression occurring ≤6 months after the initiation of palbociclib treatment, whereas patients who achieved complete response, partial response (PR) or durable stable disease with progression-free survival >6 months were considered treatment-responsive. Thus, the present study was designed as a predictive biomarker study based on pretreatment plasma samples rather than a cross-sectional analysis of established resistance.

Blood samples were collected into K_2EDTA anticoagulant tubes, and plasma separation was performed <2 h after collection. Briefly, 5 ml of whole blood was gently inverted several times immediately after collection and centrifuged at 1,200 x g for 10 min at 4˚C. The supernatant was collected as plasma and further centrifuged at 14,000 x g for 10 min at 4˚C to remove residual cellular debris. The resulting plasma supernatant was aliquoted and stored at -80˚C until further analysis.

Plasma metabolite extraction. In total, 200 μl of plasma was pipetted into a 1.5 ml microcentrifuge tube (EP tube), followed by the addition of 800 μl of precooled (-80˚C) methanol containing an isotopically labeled internal standard (5.00 μM Met-d_3, 0.10 μM SAM-d_3, 0.05 μM SAH-d_4 and 5.00 μM HCY-d_4). The mixture was vigorously vortexed to ensure thorough homogenization and then incubated in a -80˚C freezer for 4 h. After incubation, the samples were centrifuged at 14,000 x g for 10 min at 4˚C. The resulting supernatant was transferred to a new EP tube and subjected to centrifugal concentration under 4˚C. The concentrated sample was reconstituted with 50% methanol-water (methanol-water=1:1, v/v), thoroughly vortexed and centrifuged again at 14,000 x g for 10 min at 4˚C. Finally, the metabolite-containing supernatant was transferred to a mass spectrometry vial for subsequent analysis.

LC-MS/MS detection. Metabolite detection was performed using an LC-MS/MS system equipped with an Agilent 1260 Infinity LC system and an Agilent G6460 triple quadrupole mass spectrometer (Agilent Technologies, Inc.). An electrospray ionization (ESI) source was used for metabolite ionization and detection was conducted in positive ion mode. The mass spectrometry parameters were set as follows: Capillary voltage, 4,000 V; nebulizer pressure, 20 psi; drying gas flow rate, 11 l/min; and nitrogen gas temperature, 300˚C. The multiple reaction monitoring (MRM) transitions were as follows: Methionine: 150.1>104.1/133.0; SAM: 399.1>136.2/250.2; SAH: 385.0>134.1/136.1; homocysteine: 136.1>56.2/90.1; methionine-d_3: 153.1>107.2/136.1; SAM-d_3: 402.0>136.2/250.1; SAH: 389.1>136.0/138.1; homocysteine: 140.0>60.1/94.0. A InfinityLab Poroshell 120 HILIC-Z column (2.1x100 mm; 2.7 μm) was used for metabolite separation with an injection volume of 5 μl. The column temperature was maintained at 40˚C, and the flow rate was set to 0.4 ml/min. The aqueous phase (Phase A) consisted of a 5 mM ammonium formate aqueous solution containing 0.1% formic acid, while the organic phase was 5 mM ammonium formate in acetonitrile with 0.1% formic acid. The gradient elution program used was as follows: Phase A: 10% (0 min)>10% (1 min)>30% (4 min)>10% (4.1 min)>10% (5 min).

Calibration curve and preparation of quality control (QC). Stock solutions of metabolite standards were prepared using ultrapure water and stored for subsequent use. A series of standard solutions with gradient concentrations were prepared by serially diluting the metabolite stock solutions in a 5% bovine serum albumin (BSA) solution (used as a surrogate matrix). The concentration points of the calibration curve were set as follows: methionine: 1.00, 5.00, 10.0, 25.0 and 50.0 μM; SAM: 0.05, 0.10, 0.20, 0.50 and 1.00 μM; SAH: 0.01, 0.05, 0.10, 0.25 and 0.50 μM; homocysteine: 1.00, 5.00, 10.0, 25.0 and 50.0 μM. QC samples were prepared for the methionine cycle-related metabolites, including samples at the lower limit of quantification (LLOQ) concentration, low QC (3XLLOQ), middle QC (at the mid-concentration point of the calibration curve), and high QC (at 80% of the highest concentration point of the calibration curve).

Isotopically labeled analogs of the target metabolites were used as internal standards for quantitative analysis in the present study. A precise mass of each isotopic internal standard was accurately weighed, and then dissolved in ultrapure water to prepare 100 mM stock solutions. The internal standard stock solutions were serially diluted with water containing 0.1% formic acid to obtain working internal standard solutions, which were stored for subsequent use.

Statistical analysis. Acquisition and analysis of the raw mass spectrometry data were performed using the Agilent MassHunter Workstation Qualitative Analysis Software (version B.06.00; Agilent Technologies, Inc.). The mass spectrometry data was uploaded to Metabolomics Workbench (https://www.metabolomicsworkbench.org/; study_id: ST004922) (19).

Receiver operating characteristic (ROC) analysis of the combined metabolites was performed using multivariable logistic regression analysis. A total of five metabolites (methionine, SAM, SAH, homocysteine and SAM/SAH) were included as independent variables in the logistic regression model, with treatment response status as the dependent variable. The predicted probability generated from this multivariable logistic regression model was subsequently used as the combined metabolites score for ROC analysis. ROC analysis and the Delong test were performed with IBM SPSS Statistics version 27 (IBM Corp.).

ANCOVA was performed using SPSS (version 27.0; IBM Corp.). For each metabolite, concentration was entered as the dependent variable, disease status was entered as the fixed factor and age was entered as a covariate. The adjusted means and P-values for group differences after controlling for age were calculated. Statistical significance was set at $P<0.05$ (two-sided).

The Mann-Whitney U test was applied to compare metabolite level differences among three pairs of groups: Patients with luminal breast cancer vs. HCs, patients in the early-stage vs. patients in the late-stage and patients that are palbociclib-sensitive vs. patients that are palbociclib-resistant. Similarly, ROC analysis was used to evaluate the diagnostic efficacy of the independent/combined metabolites in these three comparisons. Least absolute shrinkage and selection operator (LASSO) regression was employed to screen for metabolites and clinical variables associated with palbociclib resistance. A logistic regression model was constructed for predicting palbociclib resistance, which was further visualized via a nomogram. The performance of the prediction model was assessed using ROC curves, calibration curves and decision curve analysis (DCA). Internal validation with bootstrap resampling (500 iterations) was performed to reduce the risk of overfitting. LASSO, logistic regression and bootstrap resampling were performed using RStudio (https://posit.co/products/open-source/rstudio).

Results

Experimental workflow and clinical characteristics. The overall experimental workflow is illustrated in Fig. 1. First, patients with breast cancer and HCs were enrolled according to the inclusion criteria, with 94 patients with the luminal A subtype, 52 patients with the luminal B subtype and 36 HCs being recruited. An MS detection method for methionine cycle-related metabolites was optimized and established in

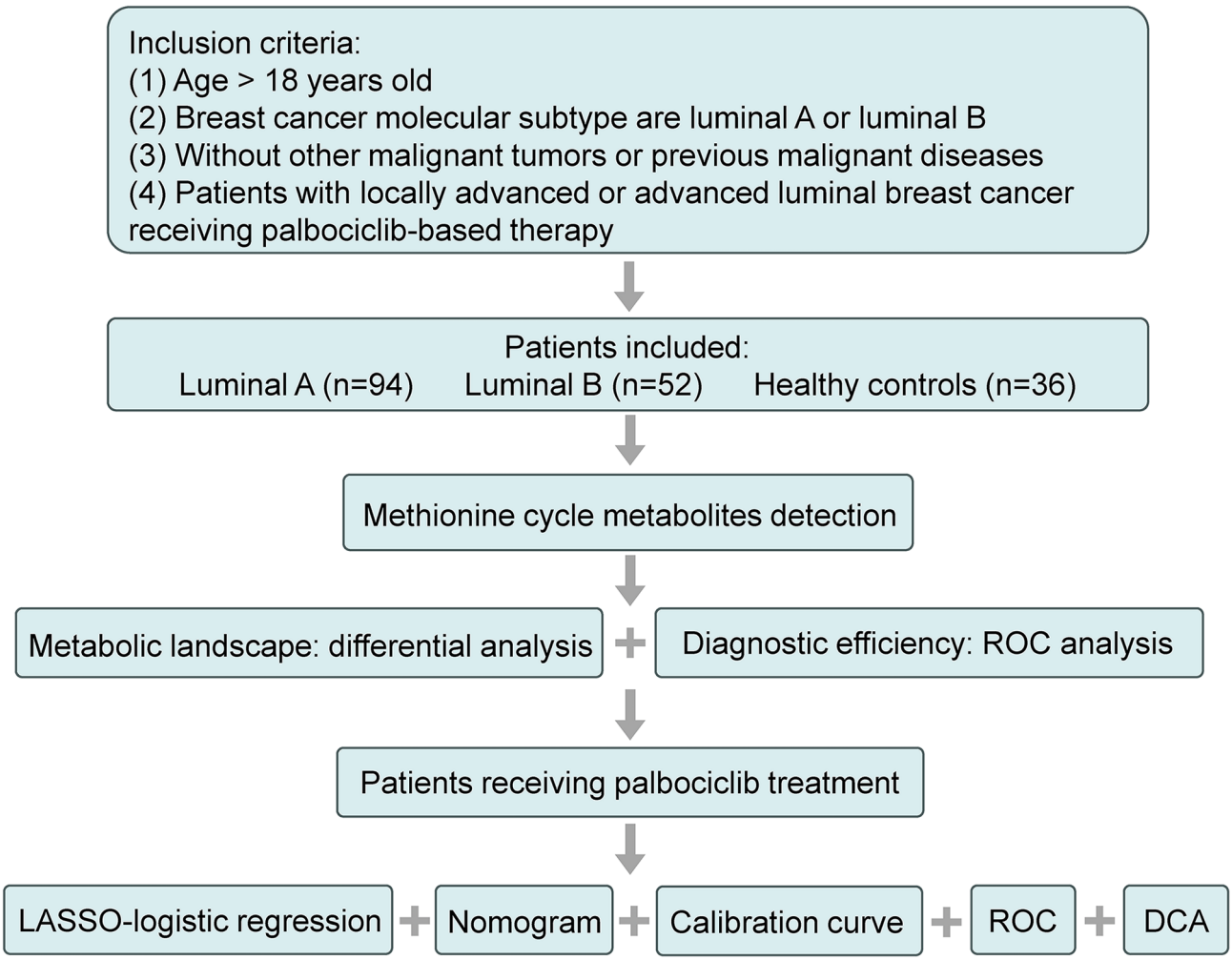

Figure 1. Experimental workflow of the present study. ROC, receiver operating characteristics; DCA, decision curve analysis; LASSO, least absolute shrinkage and selection operator.

advance. Subsequently, the metabolites were extracted from the plasma after collection and targeted MS detection was performed. Differential analysis of the metabolic landscape among groups was conducted, and the diagnostic efficacy of individual/integrated metabolites was analyzed. Subsequently, the clinical data of the patients was collected and integrated the metabolic landscape. LASSO was used to screen variables related to palbociclib resistance, and a logistic regression model was constructed and visualized with a nomogram. The performance of the prediction model was evaluated using calibration curves, ROC curves and DCA.

The clinical characteristics of the enrolled subjects are listed in Table I. The average ages of patients with luminal A/B breast cancer were 58 (31-83) years and 59 (37-82) years, respectively, and the average age of HCs was 51 (24-72) years. The differential analysis of the methionine cycle-related metabolites is presented in the subsequent sections.

Quantification of methionine cycle-related metabolites. Targeted metabolomics was employed for metabolite quantification (20,21). First, an MS detection method was established using metabolite standards. By optimizing parameters such as collision energy and fragment voltage, two MRM transitions were ultimately selected for each metabolite and its isotopic internal standard. The optimized mass spectrometry parameters are listed in Table SI. After validation, the methionine cycle-related metabolites exhibited excellent separation efficiency and mass spectrometric response within the 5-min gradient (Fig. 2A).

To achieve absolute quantification, calibration curves were constructed for the target metabolites. Specifically, calibration curves were constructed by plotting the ratio of the total MRM peak areas of target metabolites to those of their isotopic internal standards against metabolite concentrations. As shown in Fig. 2B, using a weighting factor of $1/x^2$, all the calibration curves exhibited excellent linearity within the established concentration range (R^2 >0.99). Additionally, to verify the precision and accuracy of the quantification, a quality control (QC) assessment was performed for the method. The precision of the method, including intraday and interday precision, was quantified using the percent coefficient of variation (% CV). Method accuracy was evaluated by calculating the percentage bias (% bias), which reflects the discrepancy between the measured concentration of samples and their respective standard concentrations. For all QC samples, the resulting precision and accuracy values met the acceptance criteria of ≤15% (LLOQ ≤20%). The detailed results of the QC analysis are presented in Table SII.

Table I. Clinical characteristics of the patients with breast cancer and HCs.

	Patients with breast cancer			
Clinical characteristics	Luminal A	Luminal B	HCs	Luminal vs. HCs
Sample size	94	52	36	
Age (years)	58 (31-83)	59 (37-82)	51 (24-72)	$P=2.2x10^{-2}$
TNM stage				
I	33	14		
II	34	26		
III	21	10		
IV	6	2		
Grade				
1	29	10		
2	39	24		
3	26	18		
CEA, ng/ml				
≤5	63	36		
>5	31	16		
CA125, U/ml				
≤25	67	33		
>25	27	19		
CA15-3, U/ml				
≤24	58	27		
>24	36	25		
Ki67				
<40%	52	37		
≥40%	42	15		
cfDNA, ng/ml				
<12	73	37		
≥12	211	15		
Local/distant metastasis				
No	67	40		
Yes	27	12		
Palbociclib treatment				
No	67	40		
Yes	27	12		
Methionine, μM	17.70±6.78	17.75±6.46	21.08±6.75	$P=1.2x10^{-2}$
SAM, μM	0.24±0.15	0.27±0.15	0.39±0.14	$P=2.0x10^{-6}$
SAH, μM	0.095±0.048	0.088±0.06	0.112±0.055	$P=2.5x10^{-2}$
Homocysteine, μM	8.42±4.49	8.65±4.42	10.49±4.97	$P=2.0x10^{-2}$
SAM/SAH	3.14±2.49	5.13±4.25	5.25±4.84	$P=7.7x10^{-2}$

HCs, healthy controls; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

The clinical value of the methionine cycle in luminal breast cancer. To evaluate the potential clinical value of the methionine cycle, the present study first assessed the diagnostic efficacy of metabolites for luminal breast cancer. Given the significant difference in age between the luminal group and the HCs group ($P=2.4x10^{-2}$), an analysis of covariance (ANCOVA) was performed to correct the impact of age. Compared with HCs, patients with luminal breast cancer had lower plasma concentrations of methionine ($P=1.2x10^{-2}$), SAM ($P=2.0x10^{-6}$), SAH ($P=2.5x10^{-2}$) and homocysteine ($P=2.0x10^{-2}$) (Fig. 3A). The SAM/SAH ratio, a common indicator of methylation level (22,23), did not differ ($P=7.7x10^{-2}$; Table SIII). ROC analysis of individual metabolites revealed that SAM exhibited the best diagnostic efficacy (AUC=0.7578). Notably, the combined metabolites yielded improved diagnostic results when compared with any single metabolite

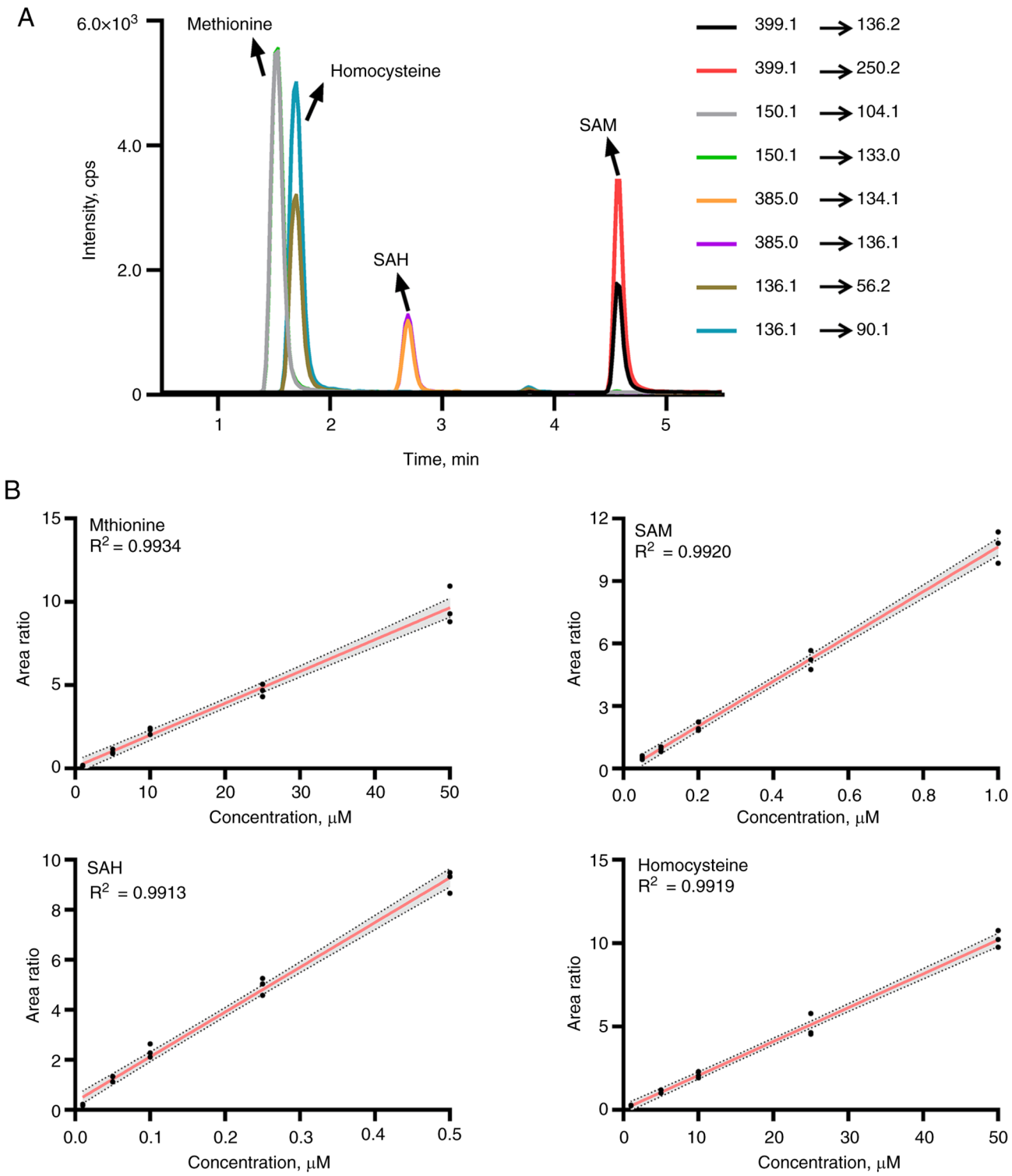

Figure 2. LC-MS/MS chromatograms and calibration curves of methionine cycle-related metabolites. (A) LC-MS/MS spectra of the metabolites. (B) Calibration curves of the metabolites. LC-MS/MS, liquid chromatography-tandem mass spectrometry.

alone (AUC=0.8130; Fig. 3B). To elucidate this difference, a DeLong test was conducted to compare the ROC curves of the combined metabolites and individual metabolites. The results revealed that the discriminatory performance of the combined metabolites was significantly improved when compared with that of the individual metabolites (Table SIV). Heatmap analysis also revealed a marked difference in metabolic landscape between patients with luminal breast cancer and HCs (Fig. S1).

To further investigate changes in the methionine cycle during the progression of luminal breast cancer, metabolite levels were compared between patients at the early-stage and late-stage. As shown in Fig. 4A, methionine ($P<0.001$), SAM ($P<0.001$), SAH ($P<0.05$) and the SAM/SAH ($P<0.05$) ratio were downregulated in patients at the late-stage, whereas homocysteine abundance did not vary with disease progression. ROC analysis revealed that SAM had the optimal performance

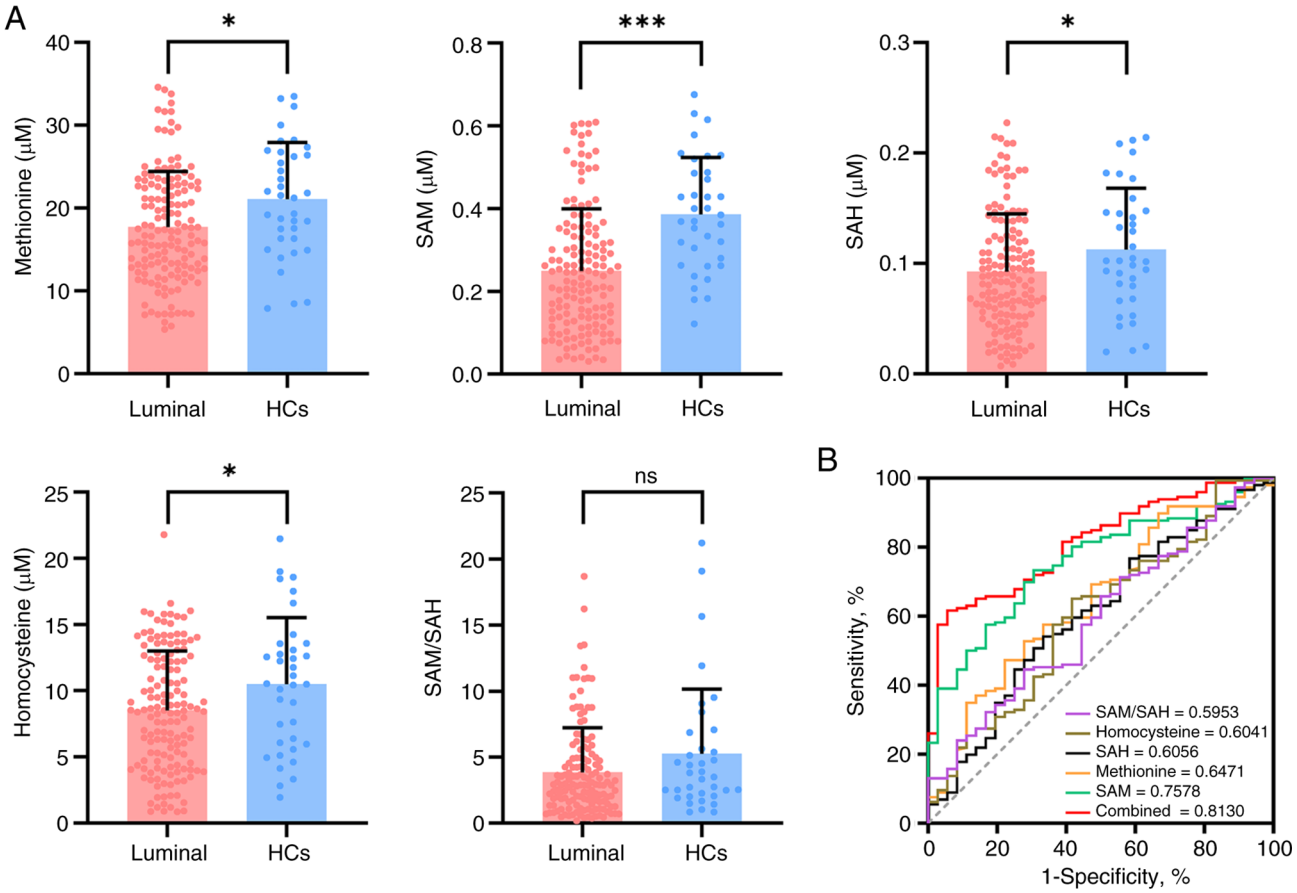

Figure 3. Metabolite concentrations and diagnostic performance in luminal breast cancer patients and HCs. (A) Metabolite concentrations in patients with luminal breast cancer and HCs (after correcting for age using ANCOVA). (B) Metabolite concentration-based receiver operating characteristic curve analysis for distinguishing patients with luminal breast cancer from HCs. HCs, healthy controls. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine. *P<0.05, ***P<0.001, ns P>0.05.

in distinguishing between early-stage and late-stage patients with luminal breast cancer (AUC=0.8514), and the combined use of multiple metabolites resulted in an even greater diagnostic AUC of 0.9228 (Fig. 4B, Table SIV). Finally, to assess the value of the methionine cycle in palbociclib resistance, the present study compared the abundance of metabolites between patients who are palbociclib-sensitive and palbociclib-resistant. The abundances of SAM, SAH and homocysteine were lower in resistant patients, whereas methionine levels and the SAM/SAH ratio did not significantly differ between sensitive and resistant patients (Fig. 5A). ROC analysis demonstrated that the diagnostic efficacy of SAM (AUC=0.7029), SAH (AUC=0.7400) and homocysteine (AUC=0.7457) was favorable, and the combination of all metabolite indicators achieved an AUC of 0.9229 for distinguishing palbociclib-sensitive/resistant patients (Fig. 5B, Table SIV).

Construction of a prediction model for palbociclib resistance. Given the limitations of current clinical methods for determining therapeutic resistance, the present study aimed to construct a palbociclib-resistance prediction model by combining the methionine cycle with clinical information. A total of 39 palbociclib-treated patients (25 sensitive vs. 14 resistant) were included in the model construction. After collecting clinical data, the correlations between the levels of methionine cycle-related metabolites and clinical characteristics were first analyzed. As shown in Fig. S2, except for a weak correlation (r=0.15, P<0.05) between methionine and age, no significant correlations were observed between the other metabolites and clinical indicators. ANCOVA further confirmed that there was no significant linear relationship between age and metabolites. These findings indirectly indicate that these metabolic indicators differ from traditional clinical indicators.

Subsequently, patients were divided into an internal training set and an internal validation set at a 7:3 ratio based on their resistance status. The training cohort included 18 sensitive and 11 resistant patients, while the validation cohort included 7 sensitive and 3 resistant patients. Here, LASSO regression was used to screen variables associated with resistance (Fig. 6A), and 10-fold cross-validation was employed for iterative analysis (Fig. 6B). The optimal model performance was achieved when the minimum criterion was applied (lambda.min=0.0424), with a total of 5 variables selected, including methionine, SAM, SAH, homocysteine and carbohydrate antigen 153 (CA153). On the basis of these variables, the present study developed a multivariate logistic regression model and further established a prediction model for the probability of palbociclib resistance. This prediction model was visualized as a nomogram (Fig. 6C), which calculates the probability of palbociclib resistance based on the total score corresponding to each variable. To reduce the risk of overfitting, the present

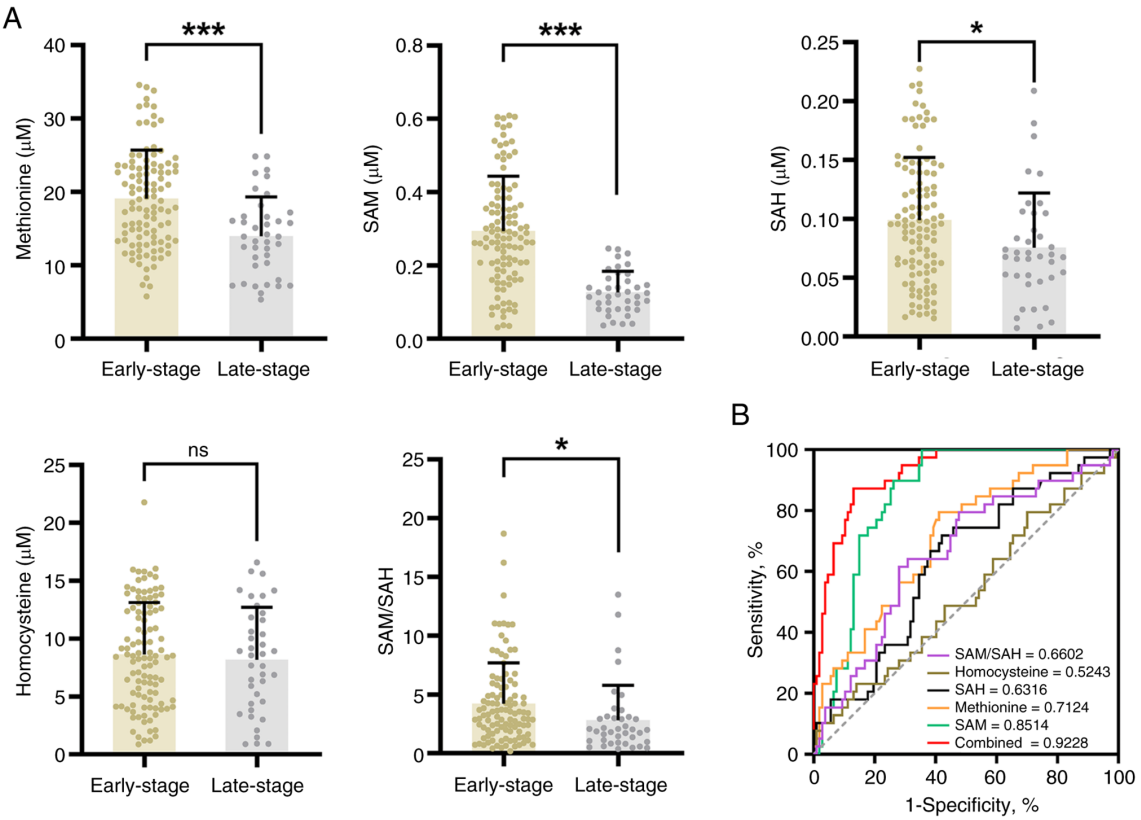

Figure 4. Metabolite concentrations and diagnostic performance in early-stage and late-stage luminal breast cancer patients. (A) Metabolite concentrations in patients with early-stage and late-stage luminal breast cancer. (B) Metabolite concentration-based receiver operating characteristic analysis for distinguishing early-stage from late-stage patients with luminal breast cancer. *P<0.05, ***P<0.001, ns P>0.05. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

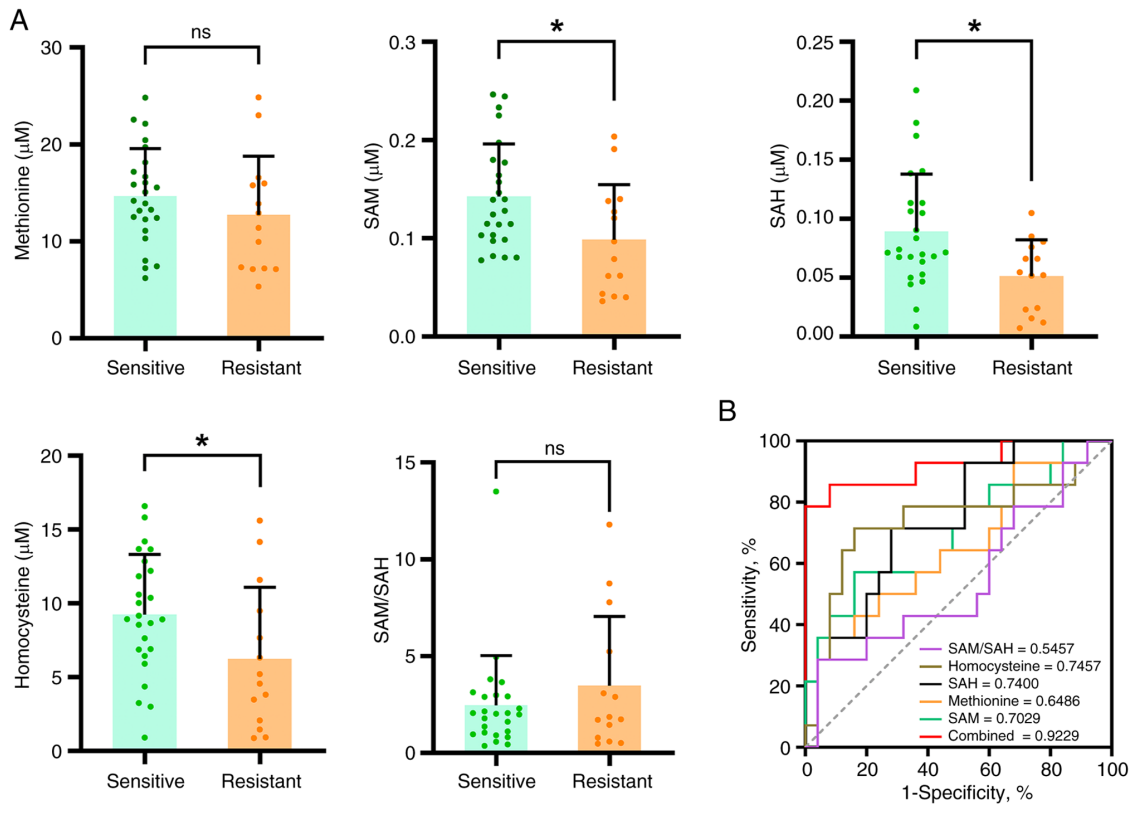

Figure 5. Metabolite concentrations and diagnostic performance in palbociclib-sensitive and palbociclib-resistant luminal breast cancer patients. (A) Metabolite concentrations in palbociclib-sensitive and palbociclib-resistant patients with breast cancer. (B) Metabolites concentration-based receiver operating characteristic curve analysis for distinguishing palbociclib-sensitive from palbociclib-resistant patients with breast cancer. *P<0.05, ns P>0.05. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

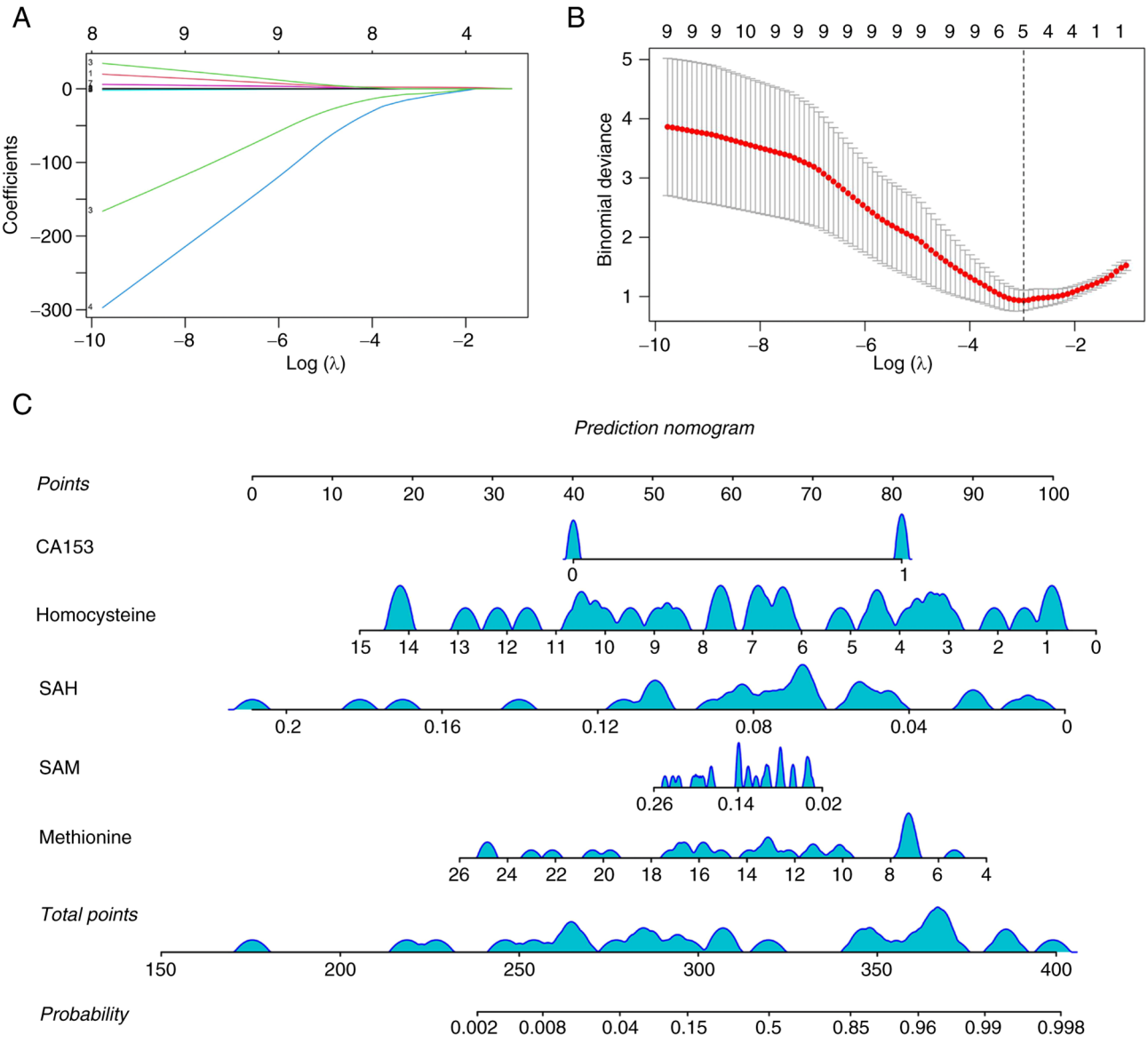

Figure 6. LASSO-based variables selection and nomogram construction for palbociclib resistance prediction. (A) The coefficient profile of least absolute shrinkage and selection operator regression. (B) 10-fold cross-validation for variable selection. (C) A prediction nomogram for predicting palbociclib resistance based on metabolites and clinical characteristics. LASSO, least absolute shrinkage and selection operator; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

study further performed internal validation using bootstrap resampling (500 iterations) for correction (Fig. S3).

Multiple metrics were employed to validate the model for accuracy and reliability. ROC analysis revealed an apparent AUC of 0.9795 (95% CI, 0.9409-1.0000) in the training set (Fig. S4). The mean optimism was 0.0724, yielding an optimism-corrected AUC of 0.9089, indicating that the model maintained good discrimination after correction. The internal validation set yielded an AUC of 0.8000, but its confidence interval was unstable because of the small sample size. The Hosmer-Lemeshow test (training set: $P=7.64x10^{-1}$; validation set: $P=3.57x10^{-1}$) confirmed that the correction was acceptable. Calibration curve analysis further confirmed the favorable agreement between observed and predicted results (Fig. S5). DCA demonstrated the model's clinical benefits (Fig. S6). In summary, all these results support the potential clinical utility of the prediction model.

Discussion

As a source of cellular nutrients, amino acid metabolism provides the material basis for tumor growth and proliferation. Amino acids, their metabolites and metabolic enzymes directly or indirectly affect tumor cells, influence the tumor microenvironment and regulate the host immune system, collectively controlling tumor progression. Methionine metabolism has recently become an emerging focus and potential therapeutic target in tumor metabolism research (24). As the core component of methionine metabolism, the methionine cycle has been found to be associated with metabolic dysregulation in various diseases (25,26). As the most prevalent subtype of breast cancer, luminal breast cancer is defined by core clinical features of hormone dependence and endocrine therapy sensitivity. However, the emergence of endocrine resistance and subsequent resistance

to CDK4/6 inhibitors (for example, palbociclib) remains a key bottleneck limiting improved disease prognosis. While previous studies have mostly focused on genetic variations and aberrant signaling pathways to elucidate luminal breast cancer pathogenesis (27,28), metabolic reprogramming, especially methionine cycle dysregulation, has received less attention in this subtype.

The present study, for the first time, explored the potential association between the methionine cycle and luminal breast cancer, and clarified its utility in diagnosis, disease progression and subsequent therapeutic resistance. On the basis of the variables screened via LASSO, the present study constructed a multivariate logistic regression model and a probability-prediction nomogram for palbociclib resistance, with further reliability confirmation of this strategy.

As a central branch of one-carbon metabolism, the methionine cycle regulates methylation reactions, epigenetic remodeling and redox homeostasis through the sequential conversion of methionine, SAM, SAH and homocysteine. Previous studies have reported reduced methionine-cycle activity in breast cancer cells compared with normal breast epithelial cells (29,30). Consistent with these findings, the present study observed significantly decreased plasma methionine, SAM, SAH, homocysteine levels, as well as SAM/SAH ratios in patients with luminal breast cancer, suggesting systemic suppression of methionine cycle metabolism during tumor development.

Tumor cells are known to exhibit 'methionine dependence', characterized by enhanced methionine uptake and utilization to support rapid proliferation, nucleotide synthesis and transmethylation reactions. Accordingly, methionine restriction suppresses tumor growth, whereas EMSY-driven methionine metabolism promotes breast cancer progression (31). Intermittent methionine deprivation can also enhance CD8+ T-cell-mediated antitumor immunity (32). In the present study, serum methionine levels further decreased in advanced-stage and palbociclib-resistant patients, suggesting that aggressive and resistant tumors may have greater methionine demand and metabolic consumption. As the major methyl donor *in vivo*, SAM regulates DNA, RNA and histone methylation and thereby influences gene expression and tumor progression. SAM-dependent methylation has been implicated in m6A modification and histone trimethylation during cancer metastasis (33). Increased SAM enhances SETD1A-mediated trimethylation of histones, ultimately facilitating the metastasis of colorectal cancer (34). Enhanced methyltransferase activity in tumors may accelerate SAM consumption and methyl-group turnover. Consistently, the present study found significantly decreased SAM levels in advanced-stage and resistant patients, indicating increased methylation demand and epigenetic reprogramming during tumor progression and palbociclib resistance.

SAH, the downstream product of methylation reactions, is a potent inhibitor of methyltransferases, while the SAM/SAH ratio is considered a better indicator of methylation potential than either metabolite alone. Reduced SAM/SAH ratios have been reported in patients with liver cancer and cancer cachexia (25). Recent studies have associated serum SAH levels with cancer prognosis (35,36), while cellular investigations have revealed the potential role of SAH hydrolase in tumor invasion and metastasis (37,38). In the present cohort, both SAH and the SAM/SAH ratio progressively decreased from healthy controls to advanced-stage and resistant patients, suggesting impaired methylation capacity and disrupted one-carbon metabolic homeostasis in aggressive luminal breast cancer.

Homocysteine associates methionine metabolism with folate metabolism, transsulfuration pathways and glutathione synthesis. Although the findings of previous studies on homocysteine alterations in breast cancer remain controversial (39-41), the present study observed significantly lower homocysteine levels in luminal breast cancer patients compared with HCs, particularly in advanced-stage and resistant patients. Since estrogen can suppress homocysteine levels (42,43). this may partly explain the findings in the endocrine-responsive cohort. In addition, resistant tumor cells may consume more homocysteine for glutathione synthesis and antioxidant defense under endocrine therapy and CDK4/6 inhibitor-induced oxidative stress.

Collectively, the present results demonstrated a consistent downward trend in methionine, SAM, SAH, homocysteine levels, as well as the SAM/SAH ratio from HCs to advanced-stage and palbociclib-resistant patients with luminal breast cancer. These findings suggest that progressive exhaustion of methionine cycle metabolism may accompany tumor progression and therapeutic resistance, potentially reflecting increased metabolic demand, accelerated transmethylation activity, epigenetic remodeling and oxidative stress adaptation in luminal breast cancer.

The present study has several limitations. First, this was a single-center study with a limited sample size, and external validation in an independent cohort was not performed. Therefore, the generalizability of the nomogram remains to be confirmed in future studies. This is a major limitation of the present study. Second, in terms of the breadth and depth of the metabolomic analysis, the present study focused only on four key metabolites of the methionine cycle. The methionine cycle is not isolated but interacts with other pathways, and uncollected confounders (for example, renal function, folate and B12) could not be adjusted, precluding a full understanding of global metabolic reprogramming in luminal breast cancer. Finally, in terms of mechanistic research and clinical translation, the present study primarily relied on correlation analysis of clinical samples. The specific molecular mechanisms by which methionine cycle metabolites affect luminal breast cancer progression and palbociclib resistance were not validated in cell or animal models. Thus, these methionine cycle metabolites are best viewed as potential predictive biomarkers that require validation in larger multicenter cohorts. Future work should improve research on the metabolic mechanisms of luminal breast cancer and its clinical translation through multicenter prospective cohorts, multiomics techniques and basic/clinical experiments.

Using LC-MS/MS-based targeted metabolomics, the present study systematically analyzed plasma differences in key methionine cycle metabolites (methionine, SAM, SAH and homocysteine) between patients with luminal breast cancer and healthy controls, revealing significant alterations in the majority of metabolites after adjustment for

confounders. The present study further explored the clinical value of the cycle in disease progression and palbociclib resistance, identifying distinct metabolic signatures associated with tumor aggressiveness and drug resistance. Finally, the present study developed and validated a resistance prediction model that integrates these metabolic indicators with routine clinical parameters, offering a practical tool for risk stratification in patients with luminal breast cancer. Limitations include a single-center study with a small sample size without external validation, unadjusted confounders (for example, renal function, B12) and lack of mechanistic validation beyond correlation analysis. Future studies with independent cohorts, expanded metabolomic coverage and experimental validation are needed to confirm these observations.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author. The mass spectrometry data generated in the present study may be found in the Metabolomics Workbench database under accession number ST004922 or at the following URL: https://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Study&StudyID=ST004922.

Authors' contributions

FX and ZW conceived and designed the project. WW carried out the mass spectrometry experiment. CM and FX participated in acquisition of clinical data. YT, HY and YM analyzed and interpreted the data. WW, YT and ZW drafted the manuscript. FX and ZW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Blood sample collection was approved by the Ethics Committee of Jiangsu Cancer Hospital (Nanjing, China) (approval no. 2023021), and all patients and HCs provided written informed consent.

Patient consent for publication

All patients and HCs signed written informed consent forms and agreed to the publication.

Competing interests

The authors declare that they have no competing interests

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