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Association of metabolomics with PD-1 inhibitor plus chemotherapy outcomes in patients with advanced non-small-cell lung cancer

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ABSTRACT

Background Combining immune checkpoint inhibitors (ICIs) with chemotherapy has become a standard treatment for patients with non-small cell lung cancer (NSCLC) lacking driver gene mutations. Reliable biomarkers are essential for predicting treatment outcomes. Emerging evidence from various cancers suggests that early assessment of serum metabolites could serve as valuable biomarkers for predicting outcomes. This study aims to identify metabolites linked to treatment outcomes in patients with advanced NSCLC undergoing first-line or second-line therapy with programmed cell death 1 (PD-1) inhibitors plus chemotherapy.

Method 200 patients with advanced NSCLC receiving either first-line or second-line PD-1 inhibitor plus chemotherapy, and 50 patients undergoing first-line chemotherapy were enrolled in this study. The 200 patients receiving combination therapy were divided into a Discovery set (n=50) and a Validation set (n=150). These sets were further categorized into respond and non-respond groups based on progression-free survival PFS criteria (PFS≥12 and PFS<12 months). Serum samples were collected from all patients before treatment initiation for untargeted metabolomics analysis, with the goal of identifying and validating biomarkers that can predict the efficacy of immunotherapy plus chemotherapy. Additionally, the validated metabolites were grouped into high and low categories based on their medians, and their relationship with PFS was analyzed using Cox regression models in patients receiving combination therapy.

Results After the impact of chemotherapy was accounted for, two significant differential metabolites were identified in both the Discovery and Validation sets: N-(3-Indolylacetyl)-L-alanine and methomyl (VIP>1 and p<0.05). Notably, upregulation of both metabolites was observed in the group with a poorer prognosis. In the univariate analysis of PFS, lower levels of N-(3-Indolylacetyl)-L-alanine were associated with longer PFS (HR=0.59, 95% CI, 0.41 to 0.84, p=0.003), and a prolonged PFS was also indicated by lower levels of methomyl (HR=0.67, 95% CI, 0.47 to 0.96, p=0.029). In multivariate analyses of PFS, lower levels of N-(3-Indolylacetyl)-L-alanine were significantly associated with a longer PFS (HR=0.60, 95% CI, 0.37 to 0.98, p=0.041).

Conclusion Improved outcomes were associated with lower levels of N-(3-Indolylacetyl)-L-alanine in patients with stage IIIB-IV NSCLC lacking driver gene mutations, who underwent first-line or second-line therapy with PD-1 inhibitors combined with chemotherapy. Further exploration of the potential predictive value of pretreatment detection of N-(3-Indolylacetyl)-L-alanine in peripheral blood for the efficacy of combination therapy is warranted.

Statement The combination of ICIs and chemotherapy has established itself as the new standard of care for first-line or second-line treatment in patients with advanced NSCLC lacking oncogenic driver alterations. Therefore,

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Immune-checkpoint inhibitors (ICIs) combined with chemotherapy are more widely used than monotherapy and have shown better survival for advanced NSCLC patients without oncogenic driver alterations. However, valid and easily accessible predictors are still needed to find patients who are most likely to benefit from immunotherapy plus chemotherapy.

⇒ Many studies demonstrate that metabolites are associated with the progression of NSCLC. However, the relationship between metabolites and outcome in NSCLC patients receiving ICIs combined with chemotherapy remains elusive.

WHAT THIS STUDY ADDS

⇒ Our study observes a correlation between low N-(3-Indolylacetyl)-L-alanine levels and extend PFS as an independent predictor. Thus, N-(3-Indolylacetyl)-L-alanine may hold potential for predicting therapeutic efficacy in NSCLC patients receiving chemoimmunotherapy in clinical practice.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our study provides more convenient and more dynamic biomarkers for immunotherapy, and seems to work synergistically with PD-L1 expression.

identifying biomarkers that can predict the efficacy and prognosis of immunotherapy plus chemotherapy is of paramount importance. Currently, the only validated predictive biomarker is programmed cell death ligand-1 (PD-L1), but its predictive value is not absolute. Our study suggests that the detection of N-(3-Indolylacetyl)-L-alanine in patient serum with untargeted metabolomics prior to combined therapy may predict the efficacy of treatment. Compared with detecting PD-L1 expression, the advantage of our biomarker is that it is more convenient, more dynamic, and seems to work synergistically with PD-L1 expression.

INTRODUCTION

In recent years, the treatment landscape for non-small cell lung cancer (NSCLC) lacking driver gene mutations has undergone a substantial transformation with the advent of immune checkpoint inhibitors (ICIs) targeting programmed cell death protein 1 (PD-1) or its programmed cell death ligand-1 (PD-L1).[1–3] Immunotherapy has transitioned from a secondary treatment choice to a primary therapy, spanning advanced to locally advanced and even early-stage disease, and evolving from single-agent therapy to combination approaches.[4 5] Despite the notable advantages of ICIs, only a limited subset of patients, approximately 20%, experience sustained positive responses to immunotherapy, while some encounter severe adverse effects or show no response.[6] Accumulating evidence suggests that combining therapies can enhance the efficacy of immunotherapy by boosting immune system activation compared with monotherapy.[7 8] Specifically, the combination of ICIs and chemotherapy has become the standard of care for first-line or second-line treatment in patients with advanced NSCLC lacking oncogenic driver alterations.[9]

While immunotherapy progresses, it faces new challenges, particularly in identifying suitable biomarkers to predict treatment response. Various potential biomarkers have been explored thus far to identify patients more likely to respond to ICIs. Currently, the sole validated predictive biomarker is PD-L1 expression,[10 11] although with non-definitive predictive value. Generally, patients with high PD-L1 expression, defined as a tumor proportion score (TPS)≥50%, tend to exhibit better responses to immunotherapy, but clinical benefits have also been observed in patients with TPS<1%.[12] This variability can be attributed, in part, to the temporal and spatial heterogeneity of tumors.[13] Additionally, blood-based tumor mutational burden (bTMB) has emerged as a candidate biomarker for immunotherapy and can be assessed via blood tests.[14] However, there is no universally accepted cut-off value for bTMB, and the relationship between bTMB and ICI treatment efficacy appears to be non-linear.[15] In essence, higher PD-L1 expression and elevated TMB confer a higher likelihood of responding to immunotherapy, but tumor immunity is a dynamic and intricate process influenced by multiple immunosuppressive and immunostimulatory factors.[16] Predicting the efficacy of immunotherapy with a single biomarker remains challenging. Furthermore, immunotherapy in combination with chemotherapy has become standard practice in clinical settings for the treatment of patients with stage IIIB-IV NSCLC without driver mutations, even in cases of negative or low PD-L1 expression.[17 18] Consequently, there is a clear need for effective and readily accessible biomarkers for chemoimmunotherapy, making it imperative to comprehensively explore such biomarkers.

Metabolites play a crucial role in shaping the disease phenotype, resulting from a complex interplay between the genome and environmental factors.[19 20] While genomics and proteomics are well-established fields, metabolomics is an emerging omics discipline capable of qualitatively and quantitatively analyzing all low-molecular-weight metabolites within a biological system over a defined time frame. This makes it a valuable tool for investigating the connections between metabolites and disease development.[21] Numerous metabolites have been implicated in the progression of NSCLC.[22–24] However, the relationship between metabolites and outcomes in patients with NSCLC treated with ICIs combined with chemotherapy remains unclear. Additionally, blood samples represent one of the most common sample types in metabolomics due to their clinical advantages: ease of acquisition, suitability for dynamic monitoring, and minimal invasiveness. These factors highlight the potential of serum metabolites as prognostic biomarkers for patients with stage IIIB-IV NSCLC undergoing ICI-chemotherapy treatment.

Our study aimed to establish a connection between pretreatment metabolites and treatment efficacy in patients with advanced NSCLC lacking driver mutations who received first-line or second-line PD-1 inhibitors combined with chemotherapy. Specifically, we aimed to identify differential metabolites significantly associated with prognosis and subject them to multivariate analysis to pinpoint potential differential metabolic markers capable of predicting patient outcomes along with their respective thresholds. Furthermore, we compared prognostic models employing metabolome biomarkers with those based on clinical predictors.

METHODS

Patients

A retrospective analysis was encompassed involving a total of 250 patients at Shanghai Chest Hospital from January 2019 to December 2021. Among these, 200 were diagnosed with stage IIIB-IV NSCLC according to the eighth edition of tumor, node, metastases (TNM) classification for lung cancer and underwent either first-line or second-line treatment with a PD-1 inhibitor combined with chemotherapy. The remaining 50 patients, also diagnosed with stage IIIB-IV NSCLC received first-line cytotoxic chemotherapy. Among the 200 patients undergoing combination therapy, 50 were randomly assigned to the Discovery set, and 150 to the Validation set.

Inclusion criteria for both the Discovery and Validation sets were as follows: (1) confirmed diagnosis of NSCLC through pathological assessment; (2) TNM stage IIIB to IV; (3) presence of at least one measurable lesion; (4)

receipt of first-line or second-line combination chemotherapy involving a PD-1 inhibitor, with first-line therapy consisting of chemotherapy for patients receiving second-line combination treatment. For patients treated with chemotherapy alone, the inclusion criteria were: (1) confirmed diagnosis of NSCLC through pathological assessment; (2) TNM stage IIIB to IV; (3) presence of at least one measurable lesion; (4) receipt of first-line cytotoxic chemotherapy. All 250 patients were excluded if they met any of the following criteria: (1) the presence of driver gene mutations (EGFR/ALK/ROS1), and the detection technology used for genetic testing is second-generation sequencing or PCR multigene combined detection; (2) subsequent surgical intervention after medical treatment; (3) occurrence of infection within 10 days before blood collection; (4) failure to complete essential systemic examinations such as chest CT, abdominal ultrasound, bone scan, brain MRI, or positron emission tomography (PET)-CT before enrollment in lieu of the aforementioned tests; (5) presence of severe systemic chronic conditions, such as cardiovascular and cerebrovascular disorders, severe liver or kidney dysfunction, or autoimmune diseases with the potential to impact the use of immunotherapy drugs (figure 1).

The follow-up period concluded on March 31, 2023.

Study design

The 250 patients were classified into two groups based on their treatment regimens: the chemotherapy group and the combined treatment group. Within the combined treatment group, the 200 patients were randomly divided into two subsets: the Discovery set and the Validation set. Serum samples collected from all patients before treatment underwent untargeted metabolomics analysis to identify differential metabolites. Several NSCLC articles have highlighted that the progression-free survival (PFS) rate at 12 months can be a primary observed endpoint for patient outcomes.[25–27] PFS was thus used as a criterion

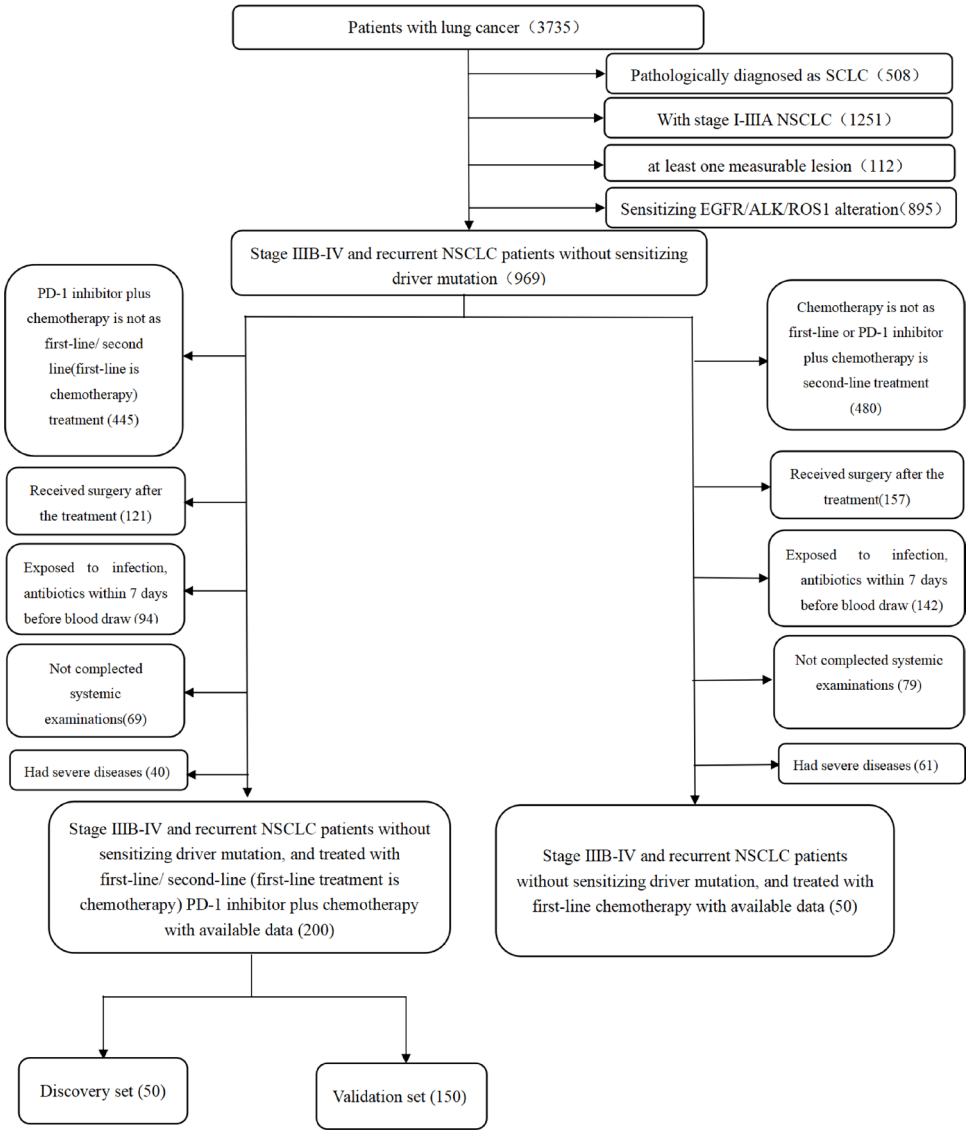

Figure 1 Inclusion and exclusion criteria for selecting patients. NSCLC, non-small cell lung cancer; PD-1, programmed cell death protein 1.

Table 1 Baseline characteristics of patients

Characteristic	Patients
Total number	250
Age (years), n (%)	
<65	104 (41.6)
≥65	146 (58.4)
Gender, n (%)	
Male	211 (84.4)
Female	39 (15.6)
Smoking history	
Never	58 (23.2)
Current/former	192 (76.8)
Histology, n (%)	
Squamous	129 (51.6)
Non-squamous*	121 (48.4)
TNM stage, n (%)	
IIIB-IIIC	76 (30.4)
IV	174 (69.6)
ECOG PS, n (%)	
0–1	206 (82.4)
2	44 (17.6)
Number of metastatic organs, n (%)	
0–1	175 (70.0)
≥2	75 (30.0)
N stage, n (%)	
0–1	51 (20.4)
2–3	199 (79.6)
PD-L1 expression, n (%)	
TPS<1%	67 (26.8)
1%≤TPS≤49%	70 (28.0)
TPS≥50%	48 (19.2)
Unknown	65 (26.0)

*Non-squamous tumor included adenocarcinoma, lymphoepithelioma-like carcinoma, adenosquamous carcinoma.
ECOG, eastern cooperative oncology group; PD-L1, programmed cell death-ligand 1; PS, performance status; TNM, tumor, node, metastases; TPS, tumor proportion score.

to further categorize patients into the non-response (NR) group (PFS<12 months) and the response (R) group (PFS≥12 months) within both the Discovery and Validation sets. By comparing the NR and R groups in the Discovery set, potential prognostic markers for the efficacy of combination therapy were sought and subsequently validated in the Validation set.

Based on the relative content of the identified differential metabolites, patients were segmented into two groups: a high group (above the median relative content) and a low group (below the median relative content). This analysis included all patients who received PD-1 inhibitor plus chemotherapy. The primary endpoint of this study was PFS, and the secondary endpoint was the objective response rate (ORR), defined as the proportion of patients with complete responses (CR) and partial responses (PR). PFS was defined from the start of treatment until the patient's disease progression or death or the last follow-up time. Tumor R was assessed by a radiologist and a clinician according to response evaluation criteria in solid tumors (RECIST) (V.1.1).

Data collection and treatment

Clinical data and laboratory parameters, including age and sex, as well as smoking history, tumor histology, TNM stage, eastern cooperative oncology group (ECOG) performance status (PS), therapeutic line, number of metastatic organs, N stage and PD-L1 expression, were retrieved from medical records. Monitoring during treatment was regularly performed, with assessments conducted every two to three treatment cycles through laboratory tests and imaging studies such as chest CT, abdominal ultrasound, and, when necessary, brain MRI, bone scan, or PET-CT.

Immunotherapy was administered as follows: intravenous administration of pembrolizumab, tislelizumab, or sintilimab at a dose of 200 mg every 3 weeks. Combination chemotherapy drugs, tailored to individual patient conditions, included pemetrexed, paclitaxel/nab-paclitaxel, vinorelbine, docetaxel, gemcitabine and platinum. A similar treatment regimen was followed by chemotherapy patients, who were also treated every 3 weeks. Treatment was continued until disease progression, intolerable adverse reactions, or death. Blood samples were collected from patients within 10 days before initiating combination therapy or chemotherapy, with serum being used for untargeted metabolomics analysis.

Sample preparation and extraction for untargeted metabolomics

Serum samples were retrieved from the −80°C freezer and thawed on ice until no ice was present. All subsequent procedures were performed on ice. Following thawing, a 10 s vortex was applied to each sample, and 50 µL of the sample was transferred to centrifuge tubes labeled accordingly. Subsequently, 300 µL of a 20% acetonitrile methanol internal standard extract was introduced into each tube, followed by a 3 min vortex. The samples underwent centrifugation at 12,000 r/min for 10 min at 4°C. Following centrifugation, 200 µL of the resulting supernatant was meticulously transferred to separate labeled centrifuge tubes, allowing them to stand in a −20°C freezer for 30 min. Subsequent to this, the samples underwent another centrifugation at 4°C and 12,000 r/min for 3 min, and 180 µL of the resultant supernatant was transferred to the liner tube of corresponding injection vials for onboard analysis. The serum samples were analyzed onboard the machine under appropriate chromatographic and mass spectrometric conditions.

Table 2 Characteristics of the patients in the chemotherapy group, Discovery set and Validation set

Characteristic	Chemotherapy (n=50)	Discovery set (n=50)	Validation set (n=150)	P value
Age (years), n (%)				
<65	23 (46.0)	15 (30.0)	66 (44.0)	0.172
≥65	27 (54.0)	35 (70.0)	84 (56.0)	
Gender, n (%)				
Male	42 (84.0)	42 (84.0)	127 (84.7)	0.990
Female	8 (16.0)	8 (16.0)	23 (15.3)	
Smoking history				
Never	11 (22.0)	10 (20.0)	37 (24.7)	0.775
Current/former	39 (78.0)	40 (80.0)	113 (75.3)	
Histology, n (%)				
Squamous	21 (42.0)	31 (62.0)	77 (51.3)	0.134
Non-squamous*	29 (48.0)	19 (38.0)	73 (48.7)	
TNM stage, n (%)				
IIIB-IIIC	18 (36.0)	16 (32.0)	42 (28.0)	0.546
IV	32 (64.0)	34 (64.0)	108 (72.0)	
ECOG PS, n (%)				
0–1	39 (78.0)	42 (84.0)	125 (83.3)	0.655
2	11 (22.0)	8 (16.0)	25 (16.7)	
Number of metastatic organs, n (%)				
0–1	35 (70.0)	38 (76.0)	102 (68.0)	0.565
≥2	15 (30.0)	12 (24.0)	48 (32.0)	
N stage, n (%)				
0–1	7 (14.0)	9 (18.0)	35 (23.3)	0.327
2–3	43 (86.0)	41 (82.0)	115 (76.7)	
PD-L1 expression, n (%)				
TPS<1%	19 (38.0)	14 (28.0)	34 (22.7)	0.654
1%≤TPS≤49%	16 (32.0)	16 (32.0)	38 (25.3)	
TPS≥50%	8 (16.0)	10 (20.0)	30 (20.0)	
Unknown	7 (14.0)	10 (20.0)	48 (32.0)	

*Non-squamous tumor included adenocarcinoma, lymphoepithelioma-like carcinoma, adenosquamous carcinoma.
ECOG, eastern cooperative oncology group; PD-L1, programmed cell death-ligand 1; PS, performance status; TNM, tumor, node, metastases; TPS, tumor proportion score.

Analysis of data

Raw data files obtained from liquid chromatography-tandem mass spectrometry (LC-MS) underwent conversion into mzXML format using Proteo Wizard software. Subsequently, XCMS program was used for peak extraction, peak sequence correction, and retention time correction. Peak areas were further adjusted employing the "SVR" method. Exclusion criteria involved peaks with less than 50% detection in each group of samples. Metabolic identification information was obtained through searches in the laboratory's self-built database, integrated public databases, artificial intelligence (AI) databases, and metDNA. For data interpretation, unsupervised principal component analysis (PCA) was conducted using R, specifically the statistical function prcomp. Data were standardized to unit variance before executing unsupervised PCA, which aimed to assess global metabolome changes between groups and ensure study stability. Supervised analysis involved orthogonal partial least squares discriminant analysis (OPLS-DA) with unit variance scaling to enhance separation between groups. Variables contributing significantly to classification were identified based on their variable importance in projection (VIP) scores. Differential metabolites were determined using criteria VIP>1 and a p value<0.05. VIP values were extracted from the OPLS-DA results, which included score plots and permutation plots generated using the R package meta-analyzer. Prior to OPLS-DA, data were log-transformed (log2) and mean-centered, and permutation testing (200 permutations) was conducted to prevent overfitting.

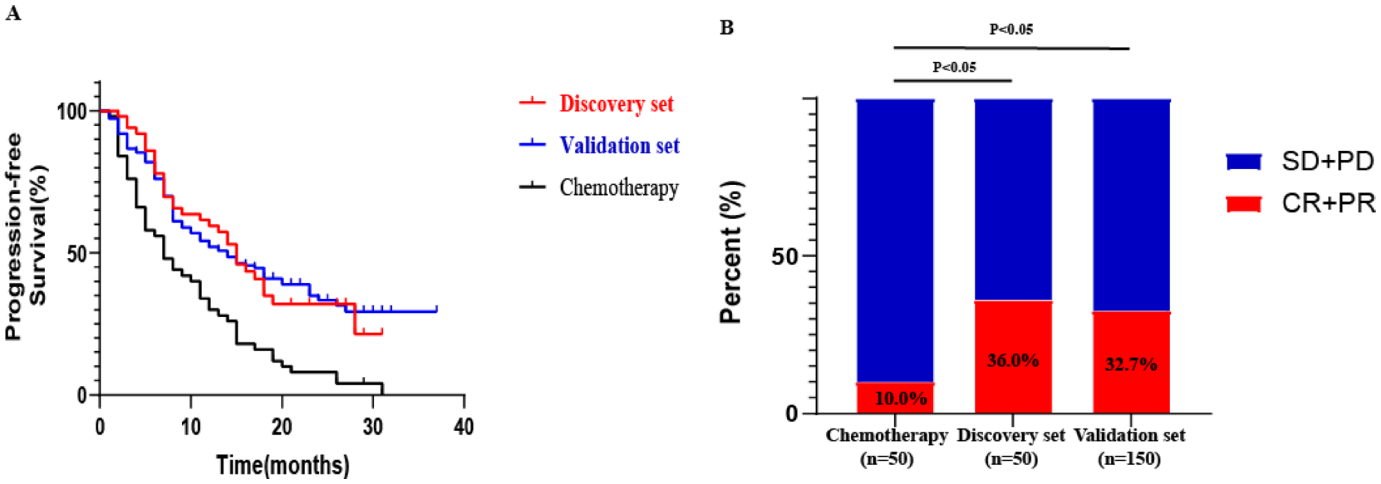

Figure 2 (A) Kaplan-Meier progression-free survival curves; (B) objective response rate of the chemotherapy group, Discovery set and Validation set. ***, p<0.05. CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

Statistical analysis of clinical data primarily used SPSS V.25.0 software and GraphPad Prism software (Prism V.8). The χ^2 test assessed the statistical significance of baseline and patient characteristic differences between groups, as well as differences in ORR. Median PFS was calculated using the Kaplan-Meier method. Cox regression analyses, encompassing univariate and multivariate analyses, determined HRs for each factor's impact on PFS. Normally distributed group comparisons were performed using t-test, while non-normally distributed group comparisons used the Wilcoxon rank-sum test. Two-sided p values<0.05 were considered statistically significant.

RESULTS

Characteristics of chemotherapy, Discovery set and Validation set

A total of 250 participants were involved in this study, and their baseline characteristics are summarized in table 1.

Among them, 200 patients (80.0%) underwent first-line or second-line combination therapy. To explore whether the detected differential metabolites were linked to immunotherapy or chemotherapy, 50 patients (20.0%) underwent first-line chemotherapy. Further categorizing patients receiving combination therapy, the Discovery set comprised 50 patients (20.0%), and the Validation set comprised 150 patients (60.0%). Table 2 demonstrates that patient characteristics were well-balanced among the three groups.

Figure 2 illustrates that the PFS was 15.0 months in the Discovery set, 18.0 months in the Validation set, and 7.0 months in the chemotherapy group. No significant differences were noted in PFS and ORR between the Discovery set and Validation set (p>0.05). However, the chemotherapy group displayed a shorter PFS and a lower ORR compared with the Discovery set and Validation set (p<0.05).

Exploring potential biomarkers

Building on the aforementioned analysis, the prognosis of the Discovery set was notably superior to that of the chemotherapy group. Subsequently, untargeted metabolomics analysis was performed on both the discovery and chemotherapy groups. The reliability of this study is evidenced by the close clustering of quality control (QC) samples in the PCA for both the chemotherapy group and the Discovery set (figure 3A). Furthermore, the OPLS-DA analysis revealed significant differences and robust predictability between the two groups (figure 3B). Initially, 1475 differential metabolites were initially selected using a combination of univariate and multivariate statistical analyses (VIP>1 and p<0.05). These were further filtered based on fold change (FC) values (FC<0.5 or FC>2), resulting in 57 metabolites of particular interest, including 4-aminobenzoate, Phe4Cl-Tyr-OH, and phenylbutazone, which exhibited the most significant differences (figure 4A). It was hypothesized that these metabolites could be linked to the prognosis of immunotherapy.

Subsequently, the focus shifted to exploring potential prognostic biomarkers for patients with advanced NSCLC treated with ICIs plus chemotherapy. Based on PFS, the Discovery set was divided into the R group (PFS≥12 months) and NR group (PFS<12 months), with balanced patient characteristics except for treatment lines, as indicated in table 3.

PCA and OPLS-DA analyses continued to demonstrate clear and reliable group separation (figure 3C & D). Using the same selection criteria as mentioned earlier, 212 differential metabolites were identified, of which 11 exhibited FC values >2 or <0.5. Among these, the metabolite Arg-Gln-Tyr-Lys showed the most significant difference (figure 4B). To account for potential interference from chemotherapy, attention was directed toward the common differential metabolites identified in two comparisons. There were 57 common differential metabolites,

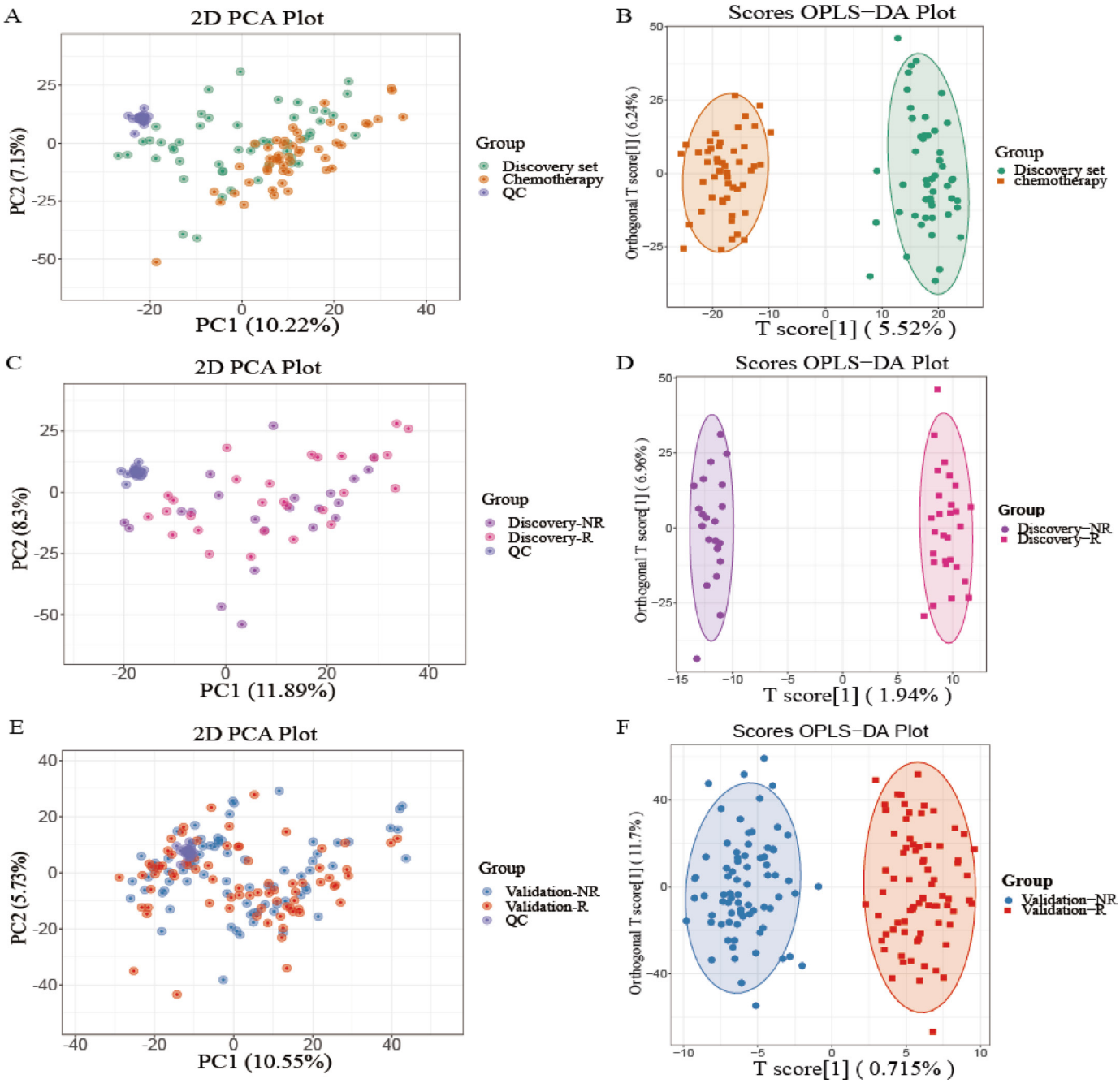

Figure 3 The principal component analysis (PCA) plot of (A) chemotherapy versus Discovery set; (C) NR group versus R group in Discovery set; (E) NR group versus R group in Validation set. The orthogonal partial least squares discriminant analysis (OPLS-DA) of (B) chemotherapy versus Discovery set; (D) NR group versus R group in Discovery set; (F) NR group versus R group in Validation set. QC, quality control; NR, non-response; R, response.

although further validation is needed to confirm whether these metabolites can serve as biomarkers (figure 5A).

Comparison of differential metabolites in Discovery and Validation sets

To validate the predictive and prognostic roles of these serum metabolites, we used an independent cohort, the Validation set, consisting of 150 samples. Similar to the Discovery set, we divided the Validation set into R and NR groups based on PFS (PFS≥12 months or <12 months) and conducted untargeted metabolomics analysis. Initially, PCA demonstrated reliability, and OPLS-DA revealed clear separations between the R and NR groups without signs of overfitting (figure 3E & F). Using the criteria of VIP>1 and $p<0.05$, we identified 150 metabolites as significant differential metabolites. The fold difference bar displayed pairwise comparisons of the top 20 annotated metabolites with the most significant FC in expression between the NR and R groups. Among these, 7-ketodeoxycholic acid exhibited the most substantial change, with an FC>2 (figure 4C).

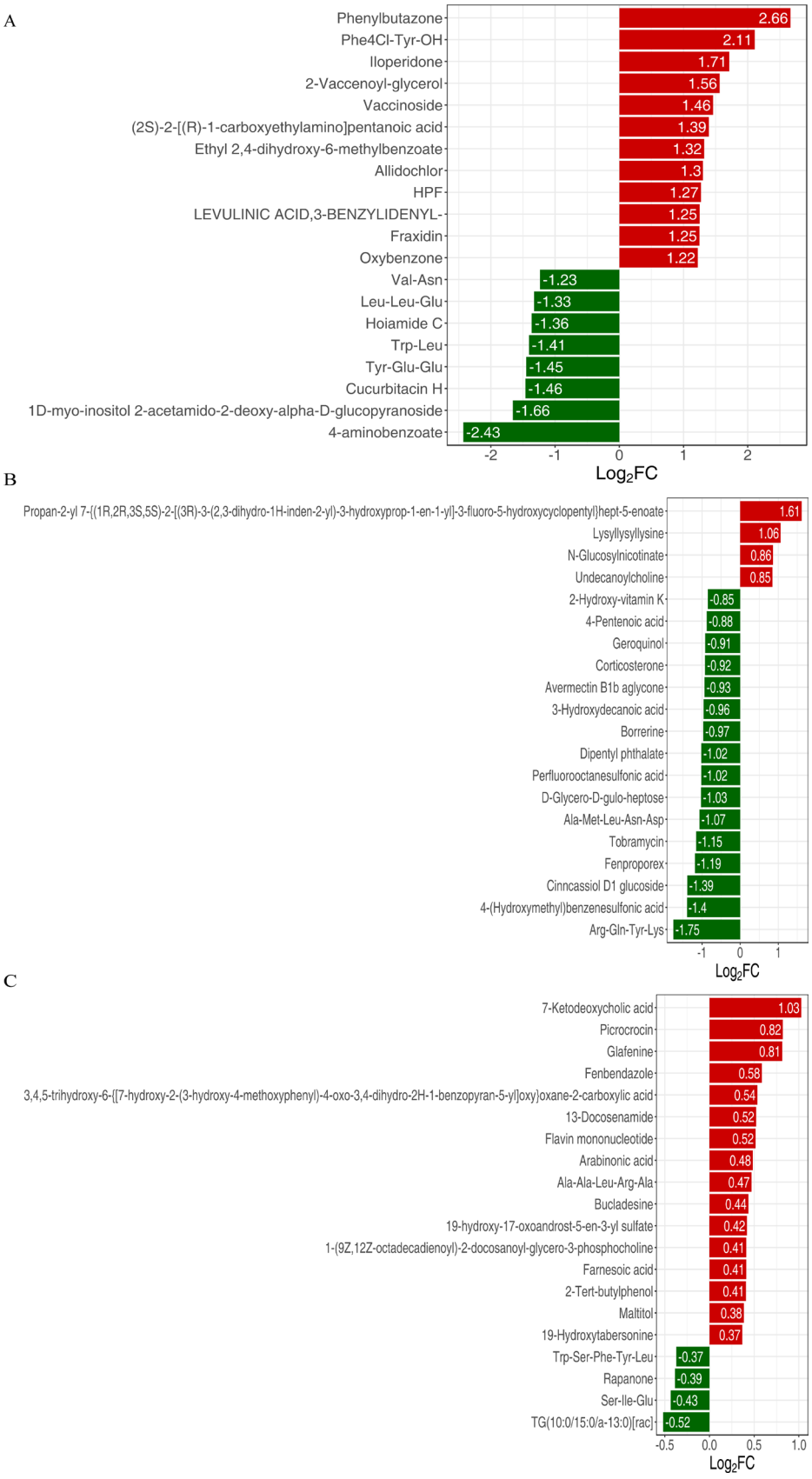

Figure 4 Fold difference bar (top 20) of (A) chemotherapy versus Discovery set; (B) NR group versus R group in Discovery set; (C) NR group versus R group in Validation set. The abscissa is the $\log_2$FC of the differential metabolite, that is, the fold difference of the differential metabolite is a logarithmic value based on 2, and the ordinate is the differential metabolite. Red represents upregulated metabolite content and green represents downregulated metabolite content. FC, fold change; NR, non-response; R, response.

Table 3 Correlation between therapeutic effect of Discovery set, Validation set and clinicopathological characteristics

	Discovery set			Validation set		
Characteristic	**NR (n=22)**	**R (n=28)**	**P value**	**NR (n=75)**	**R (n=75)**	**P value**
Age (years), n (%)						
<65	6 (27.3)	9 (32.1)	0.709	33 (44.0)	33 (44.0)	1.000
≥65	16 (72.7)	19 (67.9)		42 (56.0)	42 (56.0)	
Gender, n (%)						
Male	18 (81.8)	24 (85.7)	0.710	63 (84.0)	64 (85.3)	0.821
Female	4 (18.2)	4 (14.3)		12 (16.0)	11 (14.7)	
Smoking history						
Never	6 (27.3)	4 (14.3)	0.433	16 (21.3)	21 (28.0)	0.344
Current/former	16 (72.7)	24 (85.7)		59 (78.7)	54 (72.0)	
Histology, n (%)						
Squamous	14 (63.7)	17 (60.7)	0.833	44 (58.7)	33 (44.0)	0.072
Non-squamous*	8 (36.4)	11 (39.3)		31 (41.3)	42 (56.0)	
TNM stage, n (%)						
IIIB-IIIC	6 (27.3)	10 (35.7)	0.525	22 (29.3)	20 (26.7)	0.716
IV	16 (72.7)	18 (64.3)		53 (70.7)	55 (73.3)	
ECOG PS, n (%)						
0–1	20 (90.9)	22 (78.6)	0.428	60 (80.0)	65 (86.7)	0.273
2	2 (9.1)	6 (21.4)		15 (20.0)	10 (13.3)	
Therapeutic line, n (%)						
First-line	19 (86.4)	19 (67.9)	0.128	39 (52.0)	52 (69.3)	0.030†
Second-line	3 (13.6)	9 (32.1)		36 (48.0)	23 (30.7)	
Number of metastatic organs, n (%)						
0–1	17 (77.3)	21 (75.0)	0.852	52 (69.3)	50 (66.7)	0.726
≥2	5 (22.7)	7 (25.0)		23 (30.7)	25 (33.3)	
N stage, n (%)						
0–1	6 (27.3)	3 (10.7)	0.253	17 (22.7)	18 (24.0)	0.847
2–3	16 (72.7)	25 (89.3)		58 (77.3)	57 (76.0)	
PD-L1 expression, n (%)						
TPS<1%	5 (22.7)	9 (32.1)	0.591	21 (28.0)	13 (17.3)	0.093
1%≤TPS≤49%	6 (27.3)	10 (35.7)		19 (25.3)	19 (25.3)	
TPS≥50%	4 (18.2)	6 (21.4)		9 (12.0)	21 (28.0)	
Unknown	7 (31.8)	3 (10.7)		26 (34.7)	22 (29.4)	

*Non-squamous tumor included adenocarcinoma, lymphoepithelioma-like carcinoma, adenosquamous carcinoma.
†P value<0.05.
ECOG, eastern cooperative oncology group; NR, non response; PD-L1, programmed cell death-ligand 1; PS, performance status; R, response; TNM, tumor, node, metastases; TPS, tumor proportion score.

Comparing the Discovery and Validation groups, along with the control group (chemotherapy), we discovered three common differential metabolites shared between the Discovery and Validation sets (figure 5C). These metabolites were 3,3'-Diamino-4,4'-dihydroxydiphenyl Sulfone, N-(3-Indolylacetyl)-L-alanine, and methomyl. The VIP and p value of 3,3'-Diamino-4,4'-dihydroxydiphenyl Sulfone, N-(3-Indolylacetyl)-L-alanine, and methomyl in the Discovery set were VIP=2.25 and p=0.03; VIP=2.65 and p<0.01 and VIP=1.78 and p<0.01. The VIP and p value of 3,3'-Diamino-4,4'-dihydroxydiphenyl Sulfone, N-(3-Indolylacetyl)-L-alanine, and methomyl in the Validation set were VIP=2.01 and p=0.03; VIP=2.06 and p<0.01 and VIP=2.44 and p=0.02 (table 4).

Furthermore, we examined the variation trend in the relative content of these three metabolites. We observed that low levels of N-(3-Indolylacetyl)-L-alanine and methomyl were associated with longer PFS. However, an interesting discrepancy emerged: in the Discovery group, low levels of 3,3'-Diamino-4,4'-dihydroxydiphenyl Sulfone

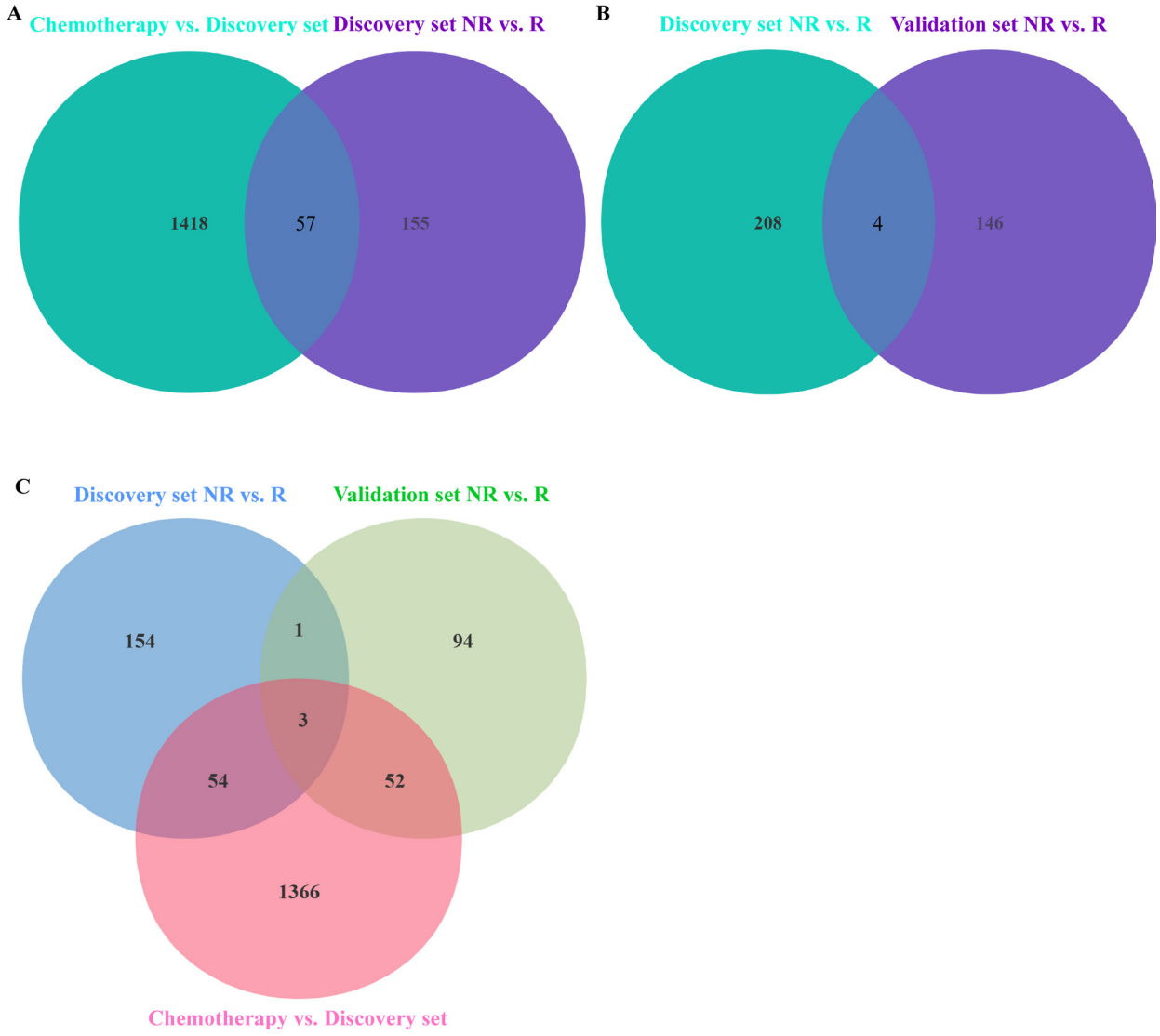

Figure 5 The Venn diagram displays the number of differential metabolites in the comparisons of (A) chemotherapy group versus Discovery set and NR versus R in Discovery set; (B) NR versus R in Discovery set and NR versus R in Validation set; (C) chemotherapy group versus Discovery set, NR versus R in Discovery set and NR versus R in Validation set. NR, non-response; R, response.

predicted better outcomes, whereas in the Validation group, low levels of 3,3'-Diamino-4,4'-dihydroxydiphenyl Sulfone predicted worse outcomes (figure 6).

These results suggest that N-(3-Indolylacetyl)-L-alanine and methomyl have the potential to differentiate patients with a favorable prognosis among advanced NSCLC cases without oncogenic driver alterations before they receive first-line or second-line PD-1 inhibitor combined with chemotherapy.

Analysis of clinical outcome on the basis of the relative content of differential metabolites

To assess the impact of the two identified metabolites, N-(3-Indolylacetyl)-L-alanine and methomyl, on the overall patient population, those receiving ICIs plus chemotherapy were divided into low and high groups according to the median relative content of these

Table 4 The common differential metabolites for comparison of three groups

	Discovery set			Validation set		
Compounds	VIP	P value	Type	VIP	P value	Type
3,3'-Diamino-4,4'-dihydroxydiphenyl Sulfone	2.25	0.03	up	2.01	0.03	down
N-(3-Indolylacetyl)-L-alanine	2.65	<0.01	up	2.06	<0.01	up
Methomyl	1.78	<0.01	up	2.44	0.02	up

VIP, variable importance in projection.

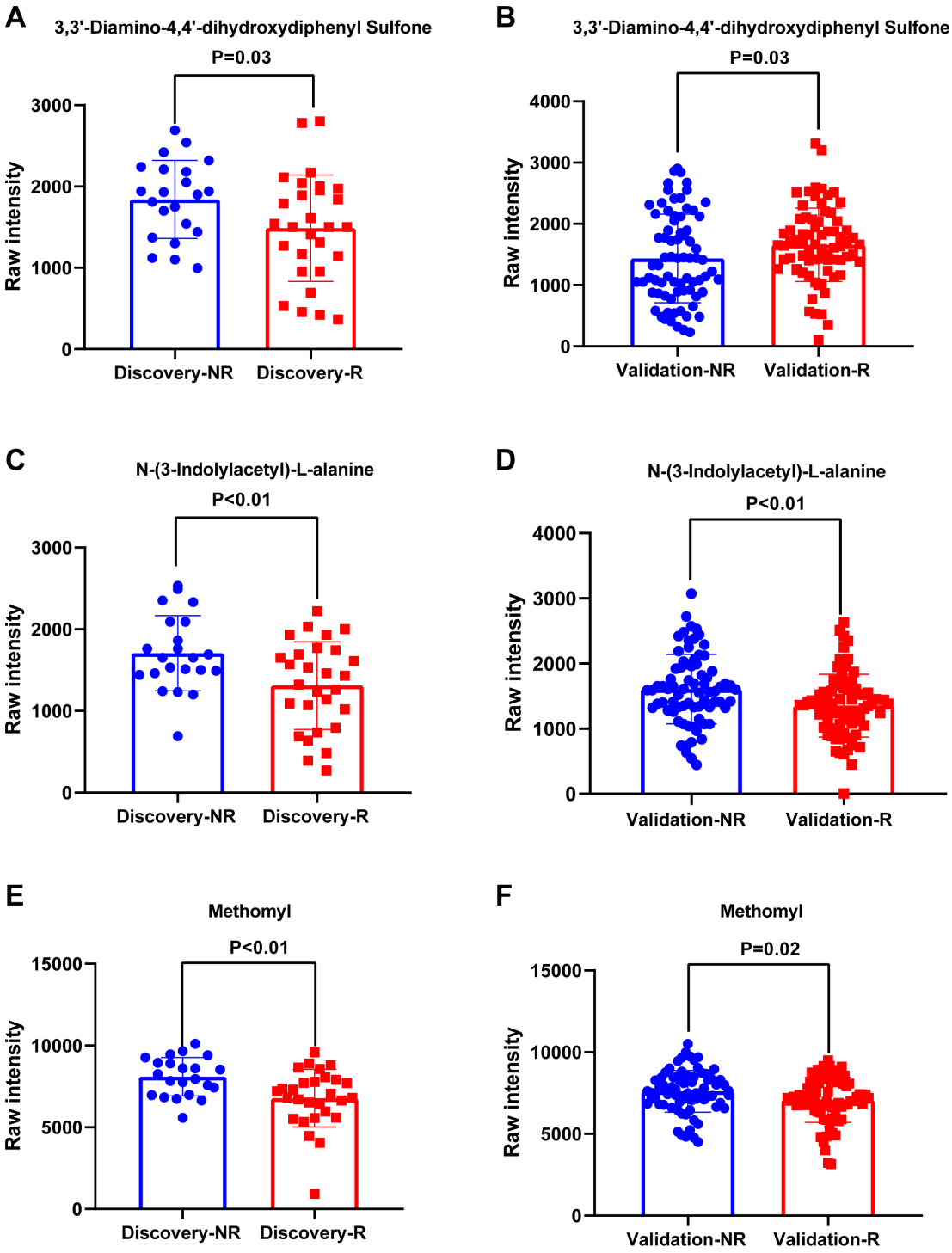

Figure 6 The histogram of (A) relative content of 3,3'-Diamino-4,4'-dihydroxydiphenyl Sulfone in Discovery set; (B) in Validation set; (C) relative content of N-(3-Indolylacetyl)-L-alanine in Discovery set; (D) in Validation set; (E) relative content of methomyl in Discovery set; (F) in Validation set. NR, non-response; R, response.

metabolites. Patient characteristics were balanced between the low and high groups for both metabolites (table 5).

The Kaplan-Meier plots in figure 7 demonstrated significant differences in median PFS between the low and high groups for both N-(3-Indolylacetyl)-L-alanine and methomyl. Specifically, the low N-(3-Indolylacetyl)-L-alanine group had a median PFS of 18.0 months, compared with 11.0 months in the high N-(3-Indolylacetyl)-L-alanine group (p=0.003). Similarly, the low methomyl group had a median PFS of 18.0 months, while the high methomyl group had a median PFS of 10.0 months (p=0.029). In a univariate analysis for PFS in table 6, no significant differences were observed with respect to patient age, sex, smoking history, histological type, TNM stage, ECOG PS, therapeutic line, number of metastatic organs and N stage.

Table 5 Correlation between the content of substance and clinicopathological characteristics

Characteristic	N-(3-Indolylacetyl)-L-alanine			Methomyl		
	Low (n=100)	High (n=100)	P value	Low (n=100)	High (n=100)	P value
Age (years), n (%)						
<65	36 (36.0)	45 (45.0)	0.195	40 (40.0)	41 (41.0)	0.885
≥65	64 (64.0)	55 (55.0)		60 (60.0)	59 (59.0)	
Gender, n (%)						
Male	84 (84.0)	85 (85.0)	0.845	83 (83.0)	86 (86.0)	0.558
Female	16 (16.0)	15 (15.0)		17 (17.0)	14 (14.0)	
Smoking history						
Never	23 (23.0)	24 (24.0)	0.868	25 (25.0)	22 (22.0)	0.617
Current/former	77 (77.0)	76 (76.0)		75 (75.0)	78 (78.0)	
Histology, n (%)						
Squamous	54 (54.0)	54 (54.0)	1.000	52 (52.0)	56 (56.0)	0.570
Non-squamous*	46 (46.0)	46 (46.0)		48 (48.0)	44 (44.0)	
TNM stage, n (%)						
IIIB-IIIC	29 (29.0)	29 (29.0)	1.000	27 (27.0)	31 (31.0)	0.533
IV	71 (71.0)	71 (71.0)		73 (73.0)	69 (69.0)	
ECOG PS, n (%)						
0–1	86 (86.0)	81 (81.0)	0.341	79 (79.0)	88 (88.0)	0.086
2	14 (14.0)	19 (19.0)		21 (21.0)	12 (12.0)	
Therapeutic line, n (%)						
First-line	68 (68.0)	61 (61.0)	0.301	65 (65.0)	64 (64.0)	0.883
Second-line	32 (32.0)	39 (39.0)		35 (35.0)	36 (36.0)	
Number of metastatic organs, n (%)						
0–1	75 (75.0)	65 (65.0)	0.123	72 (72.0)	68 (68.0)	0.537
≥2	25 (25.0)	35 (35.0)		28 (28.0)	32 (32.0)	
N stage, n (%)						
0–1	21 (21.0)	23 (23.0)	0.733	18 (18.0)	26 (26.0)	0.172
2–3	79 (79.0)	77 (77.0)		82 (82.0)	74 (74.0)	
PD-L1 expression, n (%)						
TPS<1%	21 (21.0)	27 (27.0)	0.094	22 (22.0)	26 (26.0)	0.771
1%≤TPS≤49%	24 (24.0)	30 (30.0)		30 (30.0)	24 (24.0)	
TPS≥50%	27 (27.0)	13 (13.0)		21 (21.0)	19 (19.0)	
Unknown	28 (28.0)	30 (30.0)		27 (27.0)	31 (31.0)	

The low and high groups were grouped according to the median relative metabolite content.
*Non-squamous tumor included adenocarcinoma, lymphoepithelioma-like carcinoma, adenosquamous carcinoma.
ECOG, eastern cooperative oncology group; NR, non response; PD-L1, programmed cell death-ligand 1; PS, performance status; R, response; TNM, tumor, node, metastases; TPS, tumor proportion score.

However, low N-(3-Indolylacetyl)-L-alanine indicated longer PFS (HR=0.59, 95% CI, 0.41 to 0.84, p=0.003), and low methomyl also suggested prolonged PFS (HR=0.67, 95% CI, 0.47 to 0.96, p=0.029). Furthermore, PD-L1 expression (TPS≥50%) was associated with longer PFS compared with PD-L1 expression (TPS<1%) (HR=0.33, 95% CI, 0.17 to 0.61, p<0.001). To identify independent predictors, Cox multivariate analyses were performed in figure 8. In multivariate analyses, low N-(3-Indolylacetyl)-L-alanine was significantly associated with prolonged PFS (HR=0.60, 95% CI, 0.37 to 0.98, p=0.041), while PD-L1 expression (TPS≥50%) remained associated with longer PFS (HR=0.35, 95% CI, 0.18 to 0.67, p=0.002). No significant association between methomyl and PFS was identified.

In addition to PFS, the study also evaluated the ORR. A total of 67 (33.5%) patients achieved an objective response. ORRs were 43.0% in the low N-(3-Indolylacetyl)-L-alanine group and 24.0% in the high N-(3-Indolylacetyl)-L-alanine group (figure 9A). Similarly, ORRs were 42.0%

Table 6 Univariate analysis for PFS

Variable	Univariate	
	HR (95% CI)	P value
Age (<65 vs ≥65)	0.90 (0.63 to 1.29)	0.564
Gender (male vs female)	0.95 (0.59 to 1.52)	0.832
Smoking history (never vs current/former)	0.89 (0.59 to 1.36)	0.600
Histology (non-squamous* vs squamous)	0.78 (0.55 to 1.11)	0.169
TNM stage (IIIB-IIIC vs IV)	0.89 (0.60 to 1.31)	0.550
ECOG PS (0–1 vs 2)	0.79 (0.50 to 1.24)	0.305
Therapeutic line (first-line vs second-line)	0.90 (0.63 to 1.30)	0.584
Number of metastatic organs (0–1 vs ≥2)	0.90 (0.62 to 1.33)	0.607
N stage (0–1 vs 2–3)	1.03 (0.67 to 1.56)	0.901
PD-L1 expression† (1%≤TPS≤49% vs TPS<1%)	0.70 (0.43 to 1.12)	0.133
PD-L1 expression† (TPS≥50% vs TPS<1%)	0.33 (0.17 to 0.61)	<0.001‡
N-(3-Indolylacetyl)-L-alanine (low vs high)	0.59 (0.41 to 0.84)	0.003‡
Methomyl (low vs high)	0.67 (0.47 to 0.96)	0.029‡

The low and high groups were grouped according to the median relative metabolite content.
*Non-squamous tumor included adenocarcinoma, lymphoepithelioma-like carcinoma, adenosquamous carcinoma.
†Only for patients with available PD-L1 expression data (patients with unknown PD-L1 expression were excluded).
‡**P value<0.05 indicates statistical significance.
ECOG, eastern cooperative oncology group; PD-L1, programmed cell death-ligand 1; PFS, progression-free survival; PS, performance status; TNM, tumor, node, metastases; TPS, tumor proportion score.

in the low methomyl group and 25.0% in the high methomyl group (figure 9B). The differences between the low and high groups for both metabolites were statistically significant (p<0.05). In summary, consistent with the results of untargeted metabolomics, low levels of N-(3-Indolylacetyl)-L-alanine and methomyl were associated with better outcomes for patients with advanced NSCLC treated with PD-1 inhibitors plus chemotherapy. However, N-(3-Indolylacetyl)-L-alanine emerged as an independent predictor with potentially better predictive value than methomyl.

Subgroup analysis of patients according to low or high N-(3-Indolylacetyl)-L-alanine

Subgroup analyses revealed that individuals with low levels of N-(3-Indolylacetyl)-L-alanine experienced notably extended PFS when compared with those with high N-(3-Indolylacetyl)-L-alanine levels. This difference was particularly pronounced among subgroups aged 65 and older, men, smokers, non-squamous histology, TNM stage IV, ECOG PS 0–1, first-line therapy, number of metastatic organs 0–1, N stage 2–3 and those with a TPS of 50% or higher (see figure 10). When we combined PD-L1 expression and the relative content of

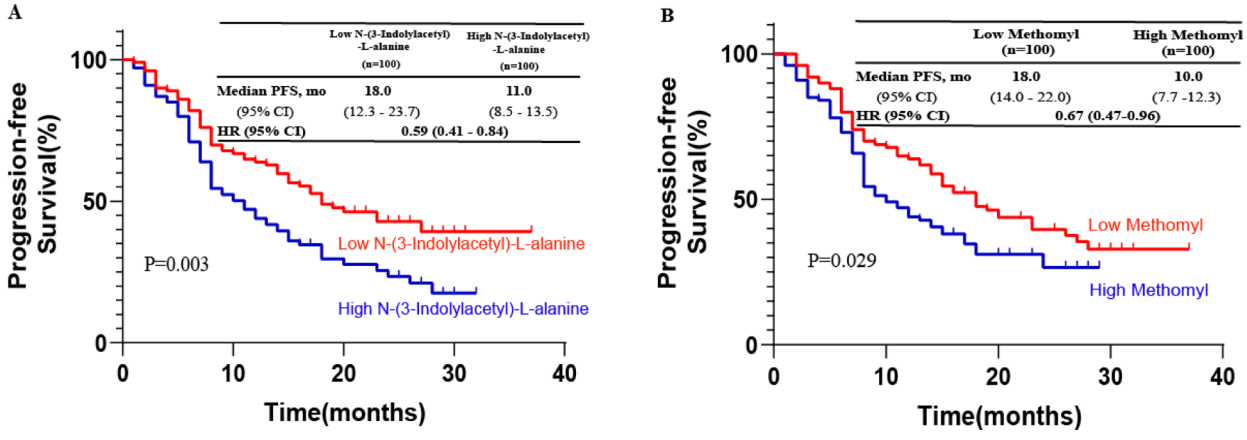

Figure 7 Kaplan-Meier progression-free survival curves according to the relative content of (A) N-(3-Indolylacetyl)-L-alanine; (B) methomyl. The low and high groups were grouped according to the median relative metabolite content. PFS, progression-free survival.

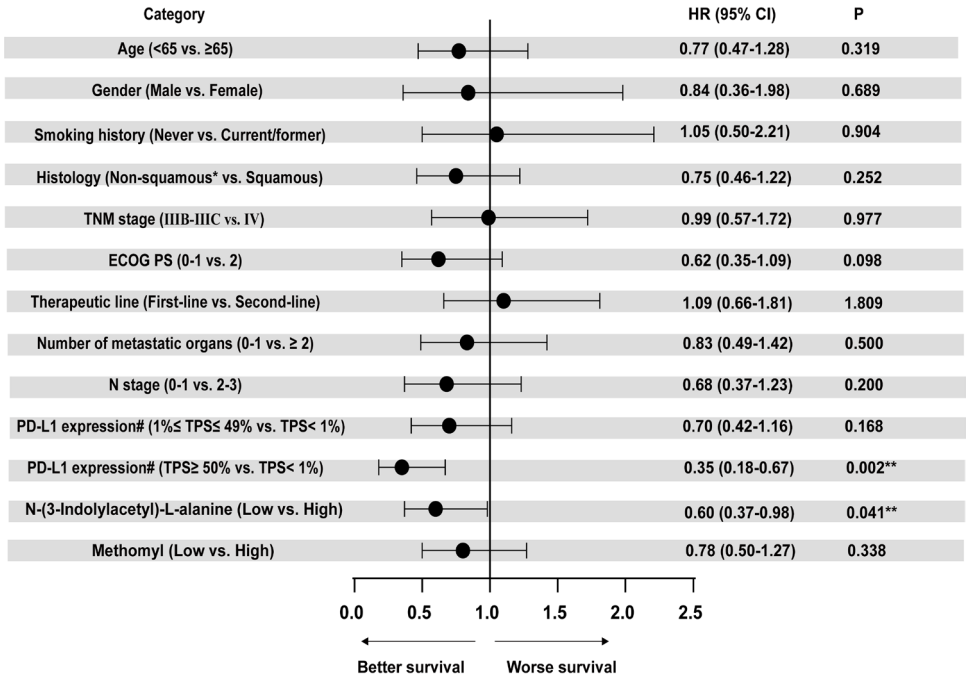

Figure 8 Multivariate Cox regression analysis of PFS. *Non-squamous tumor included adenocarcinoma, lymphoepithelioma-like carcinoma, adenosquamous carcinoma. #Only for patients with available PD-L1 expression data (patients with unknown PD-L1 expression were excluded). **, p<0.05 indicates statistical significance. The low and high groups were grouped according to the median relative metabolite content. ECOG, eastern cooperative oncology group; CR, complete response; PD, progressive disease; PD-L1, programmed cell death ligand-1; PR, partial response; PS, performance status; SD, stable disease; TNM, tumor, node, metastases.

N-(3-Indolylacetyl)-L-alanine to analyze PFS, we found that the combination of low N-(3-Indolylacetyl)-L-alanine and high PD-L1 expression (TPS≥1%) had the longest PFS, while the combination of high N-(3-Indolylacetyl)-L-alanine and low PD-L1 expression (TPS<1%) had the shortest PFS (see figure 11).

DISCUSSION

Lung cancer remains a significant public health challenge due to its high malignancy and associated morbidity and mortality rates.[28] The emergence of immunotherapy has provided new hope for patients with gene-negative lung cancer and has reshaped the treatment landscape for this disease. However, the lack of precise predictive markers has made it challenging to identify patient populations that could benefit most from immunotherapy.[29] There were many studies that illustrated metabolomics could be applied to the early detection of NSCLC. For example, researchers have detected the plasma lipid metabolome of 171 patients with early NSCLC and 140 healthy individuals, screened 9 plasma lipid markers after support vector machine algorithm and high-resolution mass spectrometry, and finally established targeted metabolic detection methods and artificial intelligence classification models. The method detected over 2,100 samples in four cohorts with more than 90% accuracy for stage I NSCLC.[23] Another study developed an NSCLC model based on metabolomic profiles in blood, and the

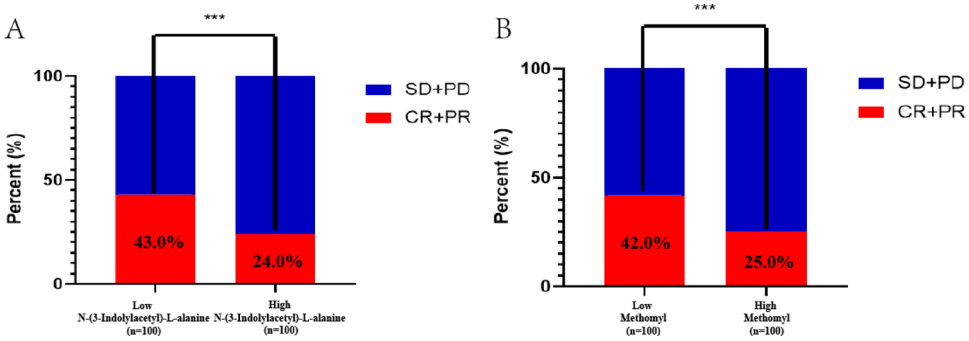

Figure 9 Therapeutic response according to the relative content of (A) N-(3-Indolylacetyl)-L-alanine; (B) methomyl. ***, p<0.05. The low and high groups were grouped according to the median relative metabolite content. CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

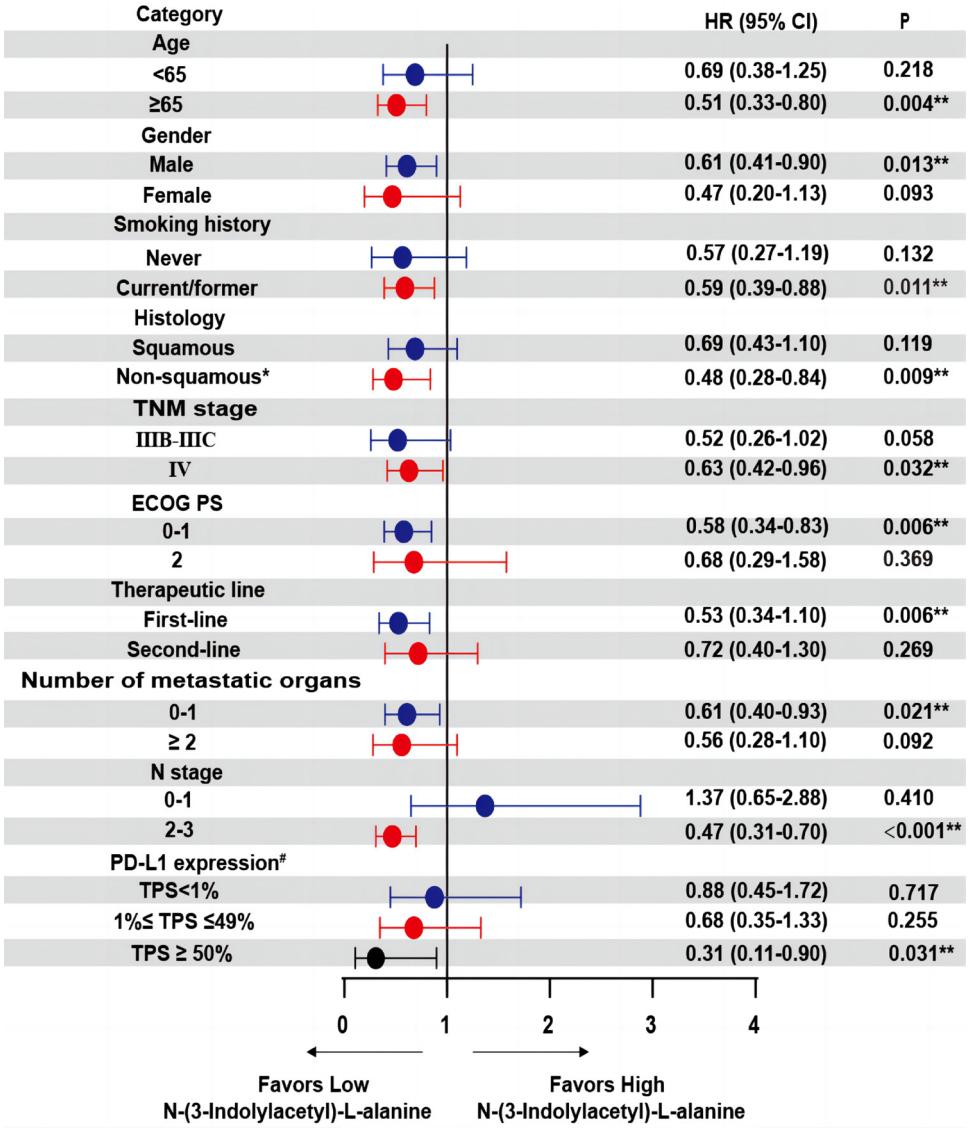

Figure 10 Subgroup analysis of progression-free survival in patients according to the relative content of N-(3-Indolylacetyl)-L-alanine. *Non-squamous tumor included adenocarcinoma, lymphoepithelioma-like carcinoma, adenosquamous carcinoma. #Only for patients with available PD-L1 expression data (patients with unknown PD-L1 expression were excluded). **, p<0.05 indicates statistical significance. The low and high groups were grouped according to the median relative metabolite content. ECOG, eastern cooperative oncology group; PD-L1, programmed cell death-ligand 1; PS, performance status; TPS, tumor proportion score; TNM, tumor, node, metastases.

researchers first validated their statistical model to identify NSCLC by measuring metabolomic profile values in blood samples from patients with NSCLC at diagnosis and comparing them with blood samples from healthy population controls. They then revalidated their model using blood samples from the same patients obtained prior to NSCLC diagnosis. The results showed that the predictive model yielded values between healthy controls and patients with NSCLC at diagnosis.[30] Additionally, metabolomics, a cutting-edge omics technology grounded in systems biology, has emerged as a promising approach for identifying effective predictive markers for lung cancer immunotherapy in the post-genomic era.[31]

Our study focused on comparing the differential metabolites between patients with good and poor prognoses in advanced NSCLC who received chemoimmunotherapy. We discovered that two metabolites, N-(3-Indolylacetyl)-L-alanine and methomyl, were significantly differentially expressed in both the Discovery and Validation sets. Notably, these metabolites exhibited consistent trends in both cohorts, with higher levels observed in patients with poor prognoses. Furthermore, low levels of N-(3-Indolylacetyl)-L-alanine were strongly associated with longer PFS in the overall population receiving combination treatment, suggesting that N-(3-Indolylacetyl)-L-alanine may be a more reliable potential biomarker compared with methomyl.

Our findings hold important implications for clinical practice. Chemoimmunotherapy is increasingly employed in the treatment of advanced NSCLC, underscoring the

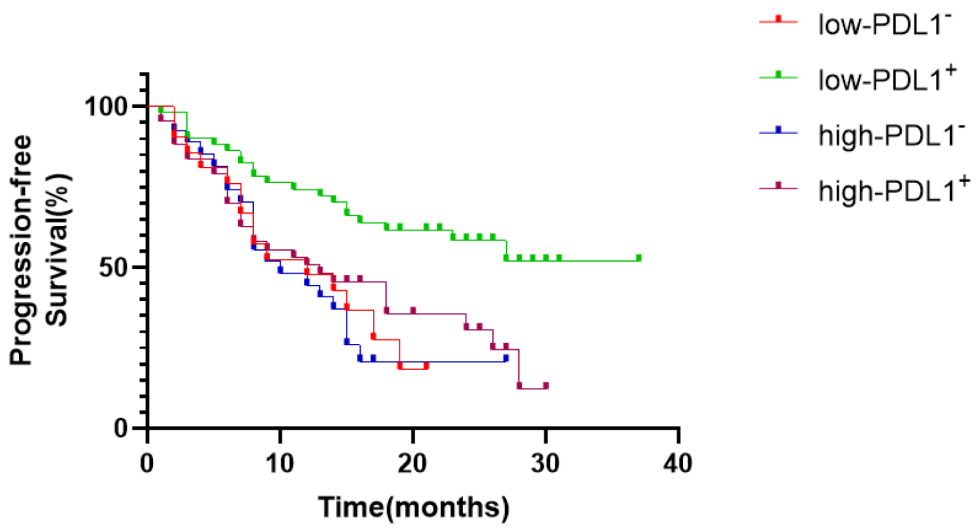

Figure 11 Kaplan-Meier progression-free survival curves according to the combination of N-(3-Indolylacetyl)-L-alanine and PD-L1 expression. The low and high groups were grouped according to the median relative metabolite content. PD-L1−: the TPS of PD-L1 expression is less than 1%; PD-L1+: the TPS of PD-L1 expression is ≥1%. PD-L1, programmed cell death-ligand 1; TPS, tumor proportion score.

need for effective biomarkers. Serum-based biomarkers offer the advantage of easy and dynamic monitoring, along with convenient and accessible testing. By employing untargeted metabolomics to analyze serum metabolites, we maximized our ability to identify relevant compounds. While previous research has explored the relationship between metabolites and the efficacy of immunotherapy in lung cancer. For example, we observed a study where authors investigated the correlation between metabolomic profiles and efficacy in patients with NSCLC using ICIs.[24] The study demonstrated alterations in 21 metabolic pathways in patients with PFS≥6 months compared with those with PFS<6 months. Notably, methane metabolism was more likely (p=0.04), while methanol metabolism was less likely (p=0.03). In another study,[32] metabolomics revealed significantly altered amino acid species in the serum of patients with lung cancer treated with PD-1 blocking antibodies, namely taurine enhanced antitumor immunity by enhancing the function of CD8+T cells. However, few studies have investigated the role of metabolites in predicting the effectiveness of ICIs combined with chemotherapy in patients with advanced NSCLC. Therefore, our study represents an innovative contribution to this field. Additionally, our use of multiple independent cohorts, including a chemotherapy group, Discovery set, and Validation set, enhances the reliability and reproducibility of our findings. Ultimately, we identified N-(3-Indolylacetyl)-L-alanine and methomyl as consistent differential metabolites in both the Discovery and Validation sets, providing valuable insights for future research and clinical applications.

N-(3-Indolylacetyl)-L-alanine is a derivative of amino acid, and although direct studies on its role are lacking, attention has grown towards the modulation of immune cell function through amino acid metabolism. Several studies have indicated that amino acid metabolism can serve as a potential therapeutic target for regulating immune responses in various conditions, including cancer, infections, and autoimmune diseases. This suggests that N-(3-Indolylacetyl)-L-alanine, as an amino acid derivative, may have relevance in the context of immune responses, making it an interesting candidate for further investigation in immunotherapy.

At present, metabolomics generally needs to be used in combination with proteomics and genomics when validating the relationship between metabolites and diseases,[33–35] which makes detection require a lot of material resources. Or the subsequent validation starts from cells or animals,[36 37] and there is still a certain gap with the real application in clinical practice. While our experiment started from real-world clinical patient biospecimens and with the help of metabolomics means, differential metabolites were analyzed to obtain, both metabolites that were found in the clinic and metabolites were verified in the clinic. We believe that biomarkers detected by metabolomics, may be unidentified metabolites, but they need to be identified in a clinical setting, which can provide clinicians with more data references when making medical decisions. To enable the utilization of metabolites as markers in clinical practice, a combination of fundamental scientific research and clinical mass spectrometry is imperative. Initial biomarker discovery occurs through untargeted metabolomics, followed by quantitative detection of metabolic markers through targeted metabolomics. Subsequently, in-depth analysis of metabolomics data with clinical information, model validation, and calibration are conducted through laboratory studies. On defining clinical value, diagnostic performance, and detection indicators, the development and production of kits ensue. Kit performance requirements mandate a linear correlation coefficient (r) greater than 0.99, a relative deviation within±15.0%, and a coefficient of variation

(CV) of ≤20.0%. Detection on references with low and high concentrations is performed, ensuring relative deviations within±15.0%. Kit application on references is repeated, and the intrabatch CV is kept at ≤15.0%. It is essential to note that laboratories need mass spectrometry and relevant pretreatment equipment for ongoing marker program validations in a clinical setting.

Instead of directly categorizing PFS, we examined the prognostic impact of these two substances within continuous data. We integrated other factors that could influence prognosis in clinical practice and incorporated metabolites into the Cox univariate and multivariate analysis model. The results revealed N-(3-Indolylacetyl)-L-alanine as an independent predictor, and lower relative levels of N-(3-Indolylacetyl)-L-alanine were linked to better outcomes. While methomyl lacked significance in the multivariate analysis, lower methomyl levels tended to correlate with improved ORR and PFS. Additionally, we conducted a subgroup analysis, showing that N-(3-Indolylacetyl)-L-alanine had a more significant predictive role in specific patient groups, like older men. As PD-L1 expression increased, the predictive capability of this metabolite gradually improved. We inferred a potential association between this metabolite and PD-L1 expression, supported by survival analysis results of their combination. Consequently, combining these two analyses is a viable approach, with metabolites dynamically monitored in the blood to compensate for limitations in PD-L1 expression detection, such as temporal and spatial variability. In summary, we validated the potential of these two metabolites as biomarkers in metabolomics and clinical applications, highlighting the strength of our study.

Despite the valuable findings produced by our study, it has certain limitations. First, it is retrospective, potentially introducing selection bias, and necessitates large prospective multicenter studies for future validation. However, the routinely collected and well-established clinical data in our study help mitigate some biases and confounding factors. For instance, regarding blood collection, we consistently obtained samples before treatment with informed patient consent, storing them under suitable conditions in hospital blood bank refrigerators at −80°C. The time and date of each blood collection and patient characteristics were also recorded in detail. After obtaining hospital ethical review consent, we obtained blood samples from patients who met the inclusion and exclusion criteria. Because the date of each blood collection was recorded, we could obtain blood samples from patients within 10 days before treatment. Second, due to insufficient maturity of overall survival (OS) data, our study predominantly focused on PFS, necessitating continuous patient follow-up to refine OS data. Efforts will be made to address the drawbacks of small sample size and lack of external validation by collaborating with multiple cancer research centers, expanding the NSCLC sample size, and increasing basic experimental studies to explore the mechanism of the study results. We have also extended metabolic and immune-related studies to other lung cancer types, such as small cell lung cancer. Furthermore, our choice of untargeted metabolomics assay for broader metabolite coverage resulted in a lack of absolute qualitative and quantitative data on the metabolites. Nonetheless, we established control and validation groups to enhance result accuracy.

In conclusion, our untargeted metabolomics approach identified and validated the upregulation of N-(3-Indolylacetyl)-L-alanine and methomyl in patients with advanced NSCLC undergoing first-line or second-line PD-1 inhibitor plus chemotherapy with poor prognoses. Additionally, a correlation between low N-(3-Indolylacetyl)-L-alanine levels and extended PFS, acting as an independent predictor was observed. Therefore, N-(3-Indolylacetyl)-L-alanine holds promise for predicting therapeutic efficacy in patients with NSCLC receiving chemoimmunotherapy in clinical practice.

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Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves human participants and was approved by Ethics Committee and Institutional Review Board of Shanghai Chest Hospital (Shanghai, China); KS23271. Participants gave informed consent to participate in the study before taking part.

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