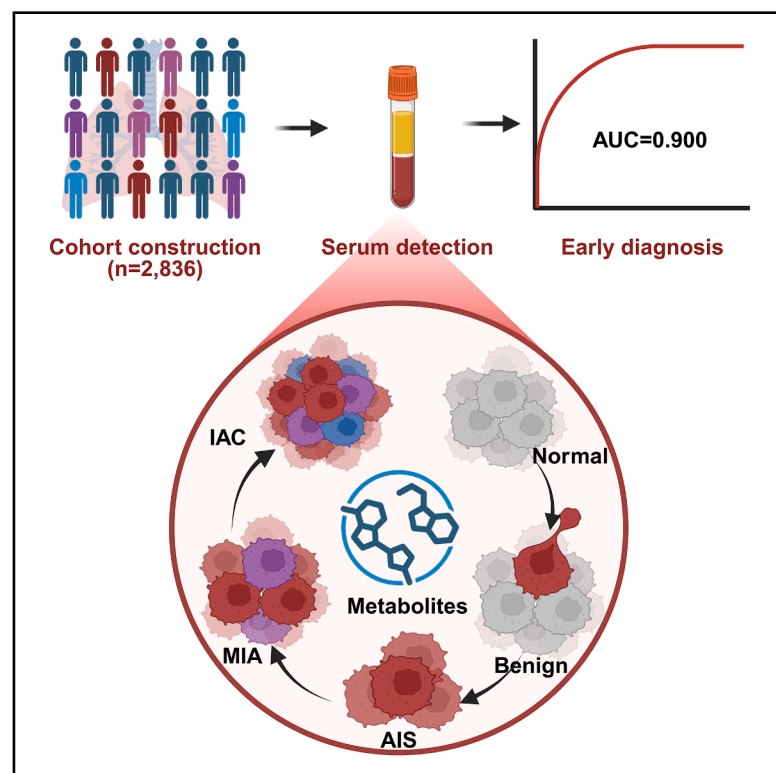


A high-resolution circulating metabolic atlas of lung adenocarcinoma progression supports early and accurate diagnosis

Graphical abstract



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In brief

Shouzhi Yang et al. map a high-resolution circulating metabolic atlas for lung adenocarcinoma progression, developing a rapid blood test to identify pre-invasive tumors and improve diagnosis accuracy.

Highlights

- Construction of a 2,836-participant retrospective and prospective serum cohort
- Resolution of a circulating metabolic atlas for lung adenocarcinoma progression
- Develops a ten-metabolite diagnostic assay, with a 0.860 AUC for pre-invasive

Article

A high-resolution circulating metabolic atlas of lung adenocarcinoma progression supports early and accurate diagnosis

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<https://doi.org/10.1016/j.xcrm.2026.102991>

SUMMARY

Early diagnosis of lung adenocarcinoma (LUAD), particularly at the adenocarcinoma *in situ* (AIS) stage, is critical for curative intervention. Profiling blood-based metabolic progression offers a systemic view of tumorigenesis to enable robust, noninvasive early detection. To achieve this, we profile 2,836 serum samples from predominantly female, never-smoking Chinese cohorts spanning normal lung to invasive carcinoma using an ultrafast nanoparticle-enhanced mass spectrometry platform. This integrated retrospective and prospective cohort design enables the construction of a comprehensive metabolic atlas, revealing conserved metabolic perturbations that arise at the pre-invasive stage and progress with histologic severity. Guided by these trajectories, we develop a ten-metabolite diagnostic assay that demonstrates reproducible performance across independent cohorts (area under the curve 0.900 for overall; 0.860 for pre-invasive disease). Notably, assay efficacy remains consistent after adjusting for demographic and lifestyle confounders. These findings identify metabolic rewiring as an early hallmark of LUAD, with potential utility in population-level early detection.

INTRODUCTION

Lung adenocarcinoma (LUAD) is the most common histological subtype of lung cancer and remains the leading cause of cancer-related mortality worldwide, accounting for approximately 1.8 million deaths annually.^{1,2} LUAD develops through a well-defined histopathological continuum, progressing from benign pulmonary lesions to adenocarcinoma *in situ* (AIS), minimally invasive adenocarcinoma (MIA), and ultimately invasive adenocarcinoma (IAC).^{3,4} Clinical outcomes are strongly stage dependent: resection of AIS is associated with cure rates exceeding 90%, whereas survival declines sharply once invasive growth is established.^{5,6} Such a stage-dependent disparity in outcomes underscores the pressing need for reliable pre-invasive detection of LUAD, to minimize the risk of overdiagnosis/overtreatment and

associated harms.^{7–9} Especially, LUAD in never-smokers is now recognized as a distinct disease entity with specific etiological and molecular characteristics, representing a major and growing global health challenge that warrants more dedicated investigation.^{10–12}

Low-dose computed tomography (LDCT) has expanded population-level lung cancer screening but generates a substantial burden of indeterminate pulmonary nodules and false-positive findings, potentially leading to prolonged surveillance or unnecessary invasive procedures.^{2,13} Tissue biopsy remains the diagnostic reference standard but carries procedural risks and is not suitable for large-scale screening or repeated assessment.¹⁴ Blood-based protein biomarkers, including carcinoembryonic antigen (CEA) and advanced multi-protein blood panel (e.g., the INTEGRAL panel), show limited sensitivity for early-stage

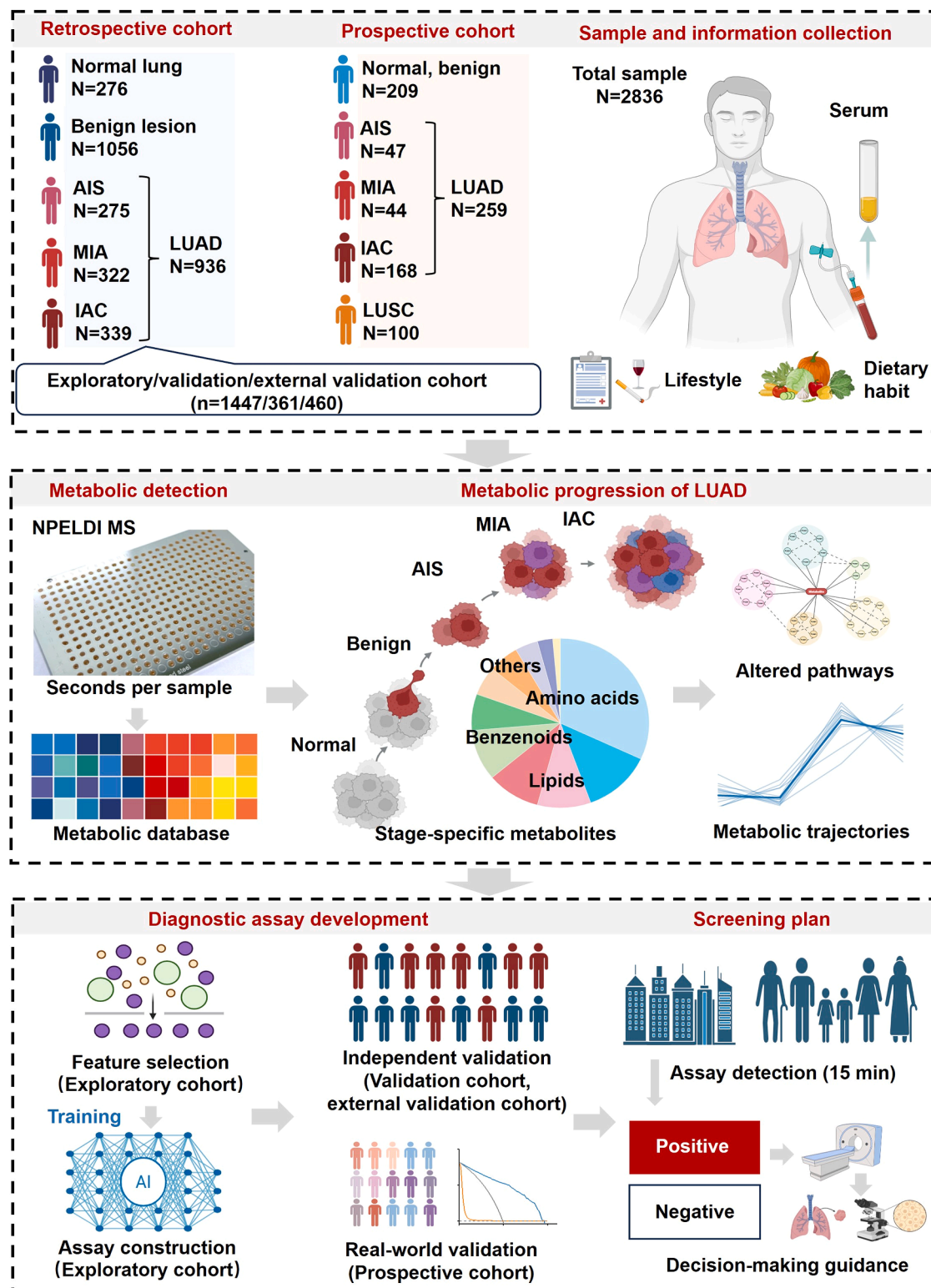


Figure 1. Overall study design

Serum samples, clinical records, lifestyle information, dietary data, and longitudinal follow-up were collected from a retrospective cohort ($n = 2,268$) and a prospective cohort ($n = 568$). Nanoparticle-enhanced laser desorption/ionization mass spectrometry (NP/ELDI-MS) was used to profile circulating metabolic changes across lung adenocarcinoma (LUAD) progression, enabling identification of altered metabolites, pathways, and metabolic trajectories. Feature selection

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disease,^{15,16} with most of advanced studies conducted predominantly in cohorts of ever-smokers. The direct applicability and performance of such biomarkers in the biologically distinct never-smoker population remain uncertain. Collectively, these limitations underscore the need for non-invasive approaches that can capture early biological changes associated with LUAD progression, particularly during the pre-invasive window and within in never-smoking population.

Large-scale molecular profiling efforts have improved understanding of LUAD biology across genomic, transcriptomic, and proteomic layers.^{17–22} However, these modalities provide indirect or delayed readouts of functional cellular state. In particular, current methylation or proteomics assays provided promising performance but also face challenges at the early stage detection of LUAD, with most exhibiting sensitivities below 40% at a fixed specificity of 98%.^{23–28} For instance, a validated cfDNA methylation-based assay reported a sensitivity of 34% for stage I cancers at 98% specificity in a cohort of 2,800 patients.²³ A proteomics assay validated across six prospective cohorts (including 624 lung cancer participants) demonstrated a sensitivity of less than 25% for stage I cancers at 98% specificity.²⁸ This performance gap underscores the need for complementary approaches that probe different biological layers.

Metabolites represent proximal outputs of cellular metabolism and microenvironmental interactions and can reflect functional changes that precede overt morphological transformation both in smoking and never-smoking individuals.^{29–33} Tissue-based metabolomic studies indicate that metabolic rewiring begins at pre-invasive stages and intensifies with disease progression, supporting metabolism as a core feature of early tumor evolution.³⁴ In contrast, the circulating metabolic landscape of LUAD progression (including never-smoking subgroups) remains incompletely characterized, in part due to technical limitations of conventional mass spectrometry (MS) and the lack of sufficiently large, stage-resolved, and well-annotated patient cohorts.³⁵

To address this unmet need, we generated a large, patient-derived, never-smoking population-predominant circulating metabolic resource spanning the full pathological continuum of LUAD. Using an ultrafast nanoparticle-enhanced laser desorption/ionization mass spectrometry (NPELDI-MS) platform, we profile 2,836 serum samples from individuals with normal lungs, benign lesions, AIS, MIA, and IAC. This dataset enables reconstruction of stage-resolved metabolic trajectories that capture early systemic metabolic alterations accompanying LUAD evolution. As a demonstration of the utility of this resource, we further derive a compact metabolic signature for LUAD detection and evaluate its performance across independent and prospective cohorts. Together, this work establishes a high-resolution circulating metabolic atlas of LUAD progression and provides a foundation for future studies of early carcinogenesis, biomarker development, and precision screening.

RESULTS

Large-scale mapping of the circulating metabolic atlas

We assembled a total of 2,836 human serum samples across three retrospective cohorts and one prospective cohort to establish a clinically representative resource for circulating metabolomic analysis (Figure 1). The retrospective cohorts comprised an exploratory cohort (714 LUAD cases and 733 non-LUAD controls), a validation cohort (192 cases and 169 controls), and an external validation cohort (30 cases and 430 controls). The prospective cohort included 259 newly diagnosed LUAD cases and 309 controls. Among them, the exploratory cohort was used to train the diagnostic model and construct the assay, while the validation cohort, external validation cohort, and prospective cohort were used to validate the assay. Cohort sizes satisfied minimum requirements established by an *a priori* power analysis. Detailed follow-up data—including dietary habits, alcohol consumption, and smoking history—were collected for all retrospective LUAD participants, enabling evaluation of lifestyle-related confounding. Age and gender distributions were comparable between cases and controls in the exploratory and validation cohorts ($p > 0.05$), minimizing potential demographic bias. Full inclusion and exclusion criteria are provided in the STAR Methods.

To characterize metabolic alterations across the LUAD disease continuum, we performed untargeted metabolomic profiling on 2,268 serum samples from the retrospective cohorts, encompassing 936 LUAD cases and 1,332 individuals with benign pulmonary lesions ($n = 1,056$) or normal lungs ($n = 276$) (Figure 2A; Figures S1–S3). All LUAD diagnoses were confirmed by histopathology and independently reviewed by two pathologists blinded to clinical information, yielding 275 AIS, 322 MIA, and 339 IAC cases (Figure 2B). Baseline clinical and lifestyle characteristics—including age, gender, smoking and alcohol use, body mass index, blood pressure, and dietary intake—were systematically documented (Figure 2C).

All samples were analyzed using an NPELDI-MS workflow, which demonstrated high sensitivity and minimal interference from the sample matrix (Figures S2A–S2E; Table S4). Notably, given the importance of MS platform reproducibility for large-scale studies, we also assessed the platform's reproducibility across a broader set of metabolites. We measured 15 typical metabolites spanning amino acids, organic acids, purines, pyrimidines, lipids, and carbohydrates. The results showed that the corresponding coefficients of variation were all below 15% (Figure S2F), meeting U.S. Food and Drug Administration requirements for *in vitro* diagnostics assay testing.³⁶

The 2,268 samples were processed in a single analytical batch across seven randomized acquisition plates to minimize inter-plate variability (Figure 2A). Pooled serum serving as quality control (QC) samples inserted every 32 runs demonstrated high

and model training were used to develop a blood-based diagnostic assay based on the exploratory cohort, which was evaluated in validation/external validation cohorts and assessed under real-world conditions using the prospective cohort. The workflow highlights the potential utility of the assay for rapid (<15 min) metabolomic profiling to support clinical decision-making. AIS, adenocarcinoma *in situ*; MIA, minimally invasive adenocarcinoma; IAC, invasive adenocarcinoma; LUSC, lung squamous cell carcinoma. See also Figure S1.

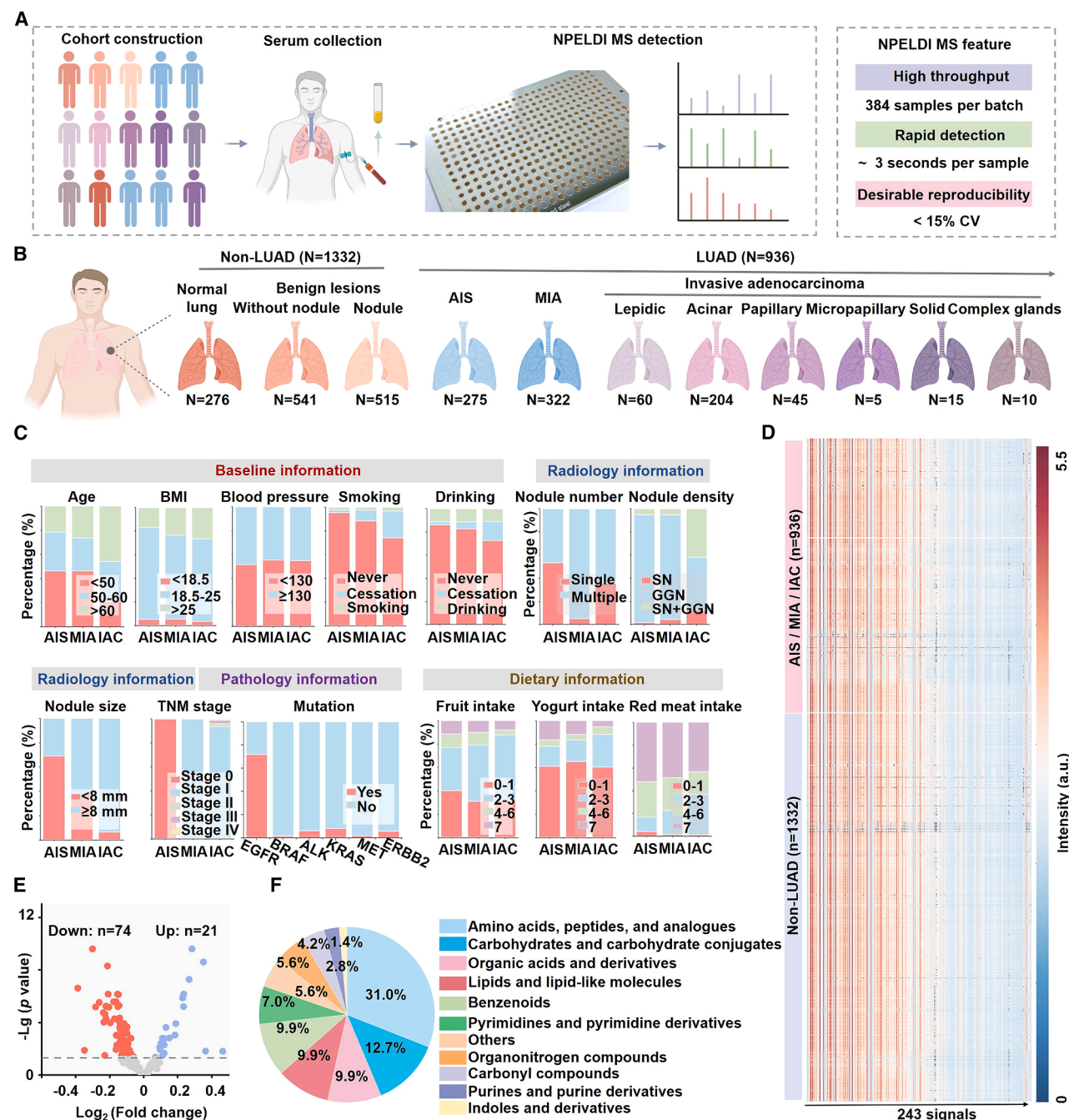


Figure 2. Large-scale mapping of the circulating metabolic atlas

(A) NPELDI-MS-based detection workflow illustrating high-throughput acquisition and analytical reproducibility.

(B) Distribution of serum samples across progressive morphological stages from normal lung to invasive adenocarcinoma.

(C) Overview of clinical and lifestyle data collected from LUAD individuals, including demographic characteristics, radiological findings, pathological diagnoses, and dietary records.

(D) Detection of 243 serum metabolic features following MS data preprocessing.

(E) Volcano plot identifying 95 differentially abundant metabolic features ($p < 0.05$, two-sided t test), including 21 increased and 74 decreased peaks in LUAD relative to non-LUAD controls.

(F) Classification of significantly altered metabolites by biochemical category. CV, coefficient of variation; BMI, body mass index; SN, solid nodule; GGN, ground-glass nodule. See also Figures S2 and S3; Tables S1–S4.

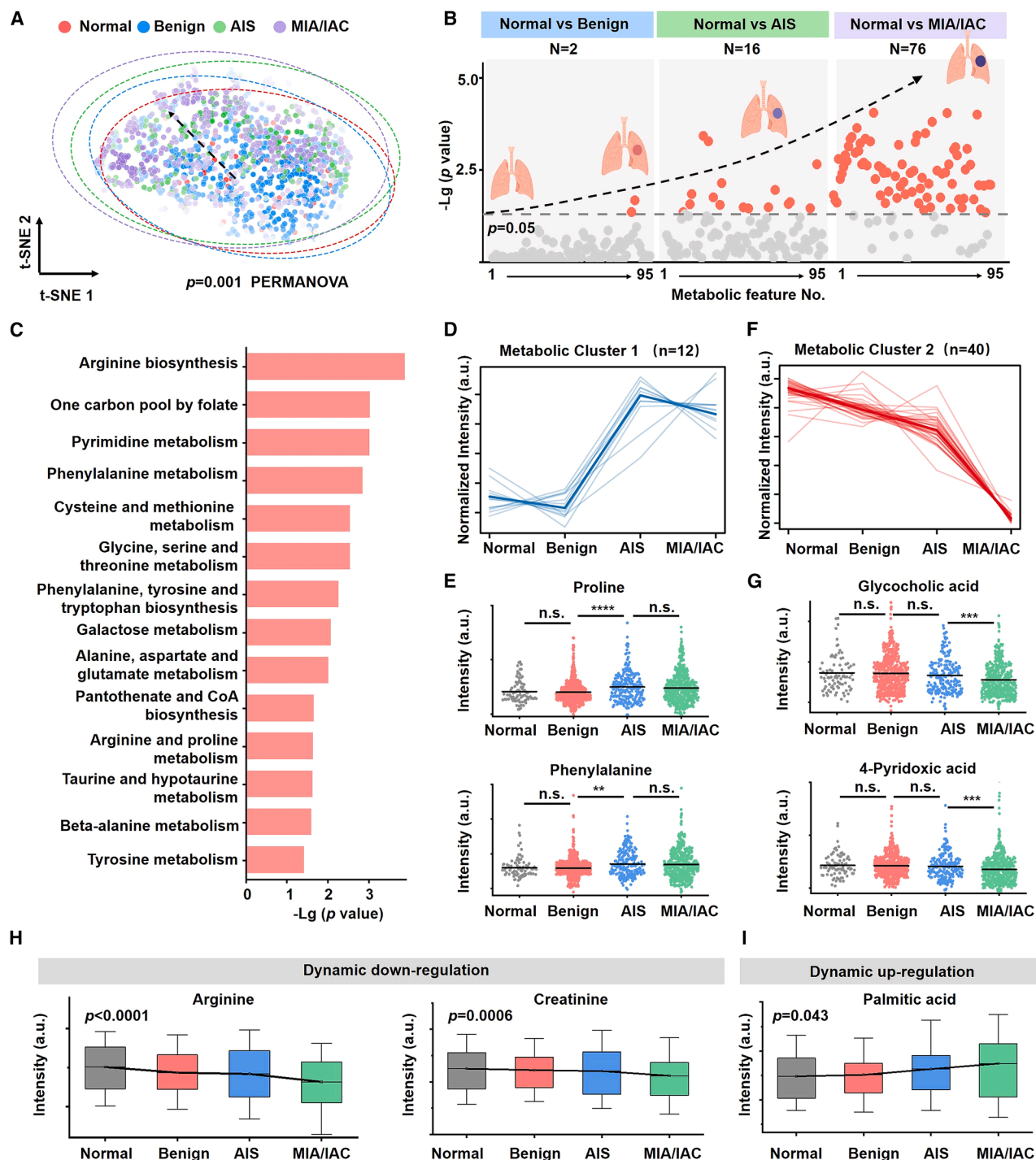


Figure 3. Metabolic evolution during LUAD progression

(A) t-distributed stochastic neighbor embedding (t-SNE) visualization of dysregulated metabolic profiles across disease stages ($p < 0.05$, permutational multivariate analysis of variance [PERMANOVA]).

(B) Progressive increase in the number of dysregulated metabolic features from normal lung to invasive LUAD ($p < 0.05$, Kolmogorov-Smirnov test).

(C) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of metabolites altered during LUAD progression.

(D) Metabolic trajectory clustering (cluster 1) showing increasing dysregulation associated with malignant transformation.

(E) Proline and phenylalanine as representative metabolites from cluster 1 ($p < 0.01$, Mann-Whitney U test). Black line refers to the mean.

(F) Metabolic trajectory clustering (cluster 2) showing decreasing pathway activity during disease progression.

(G) Glycocholic acid and 4-pyridoxic acid as representative metabolites from Cluster 2 ($p < 0.001$, Mann-Whitney U test). Black line refers to the mean.

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technical reproducibility, with a mean Pearson correlation coefficient of 0.987 and over 80% of replicates exceeding 0.980 (Figures S3A and S3B). We further calculated the coefficient of variation (CV) for each detected feature across all QC samples within the analytical batch. The mean CVs of typical metabolites, including lactic acid, phenylalanine, arginine, urea, and glutamine, were shown to be 8.43%–18.68%, supporting the reproducibility of the MS detection. Data preprocessing—including replicate averaging, baseline correction, and local maximum interpolation—generated a uniform and analytically tractable metabolomic dataset (Figure S3C).

Comparative analysis identified 95 dysregulated metabolic features (21 increased and 74 decreased; LUAD versus non-LUAD controls, $p < 0.05$; Figures 2D and 2E; Table S5), corresponding to 71 annotated metabolites spanning major biochemical classes, including amino acids and derivatives (31.0%), organic acids and derivatives (9.9%), and carbohydrates and conjugates (12.7%) (Figure 2F). Collectively, these data provide a circulating metabolic atlas that delineates metabolic changes across LUAD progression.

The circulating metabolic evolution of LUAD

Building on this dataset, we next examined how circulating metabolites change across the full histological spectrum of LUAD. Because metabolic reprogramming is a fundamental feature of early malignant transformation, we investigated whether serum profiles captured in the atlas could resolve stage-dependent metabolic evolution from normal lung and benign lesions through AIS and into minimally invasive and invasive disease.

Unsupervised visualization using both t-distributed stochastic neighbor embedding and principal component analysis demonstrated clear separation of invasive disease from benign and normal samples ($p < 0.05$, permutational multivariate analysis of variance; Figures 3A and S4A). The extent of metabolic dysregulation increased progressively with pathological severity: 2 metabolic peaks differed between benign and normal samples, 16 between AIS and normal samples, and 76 between MIA/IAC and normal samples (Figure 3B). These stepwise alterations highlight the sensitivity of blood-based metabolic profiling for detecting changes that emerge at pre-invasive stages. Notably, the annotated metabolites specifically dysregulated in AIS versus normal samples included phenylacetic acid, homocysteine, lactic acid, proline, tartaric acid, ornithine, 4-aminohippuric acid, phenylalanine, and thymidine (Table S6).

Pathway analysis based on 71 annotated altered metabolites further identified 14 altered metabolic pathways associated with early LUAD, including arginine biosynthesis, pyrimidine metabolism, and glycine/serine/threonine metabolism (Figure 3C; Table S7). Many of these changes paralleled those previously observed in tumor tissue,³⁴ whereas others—such as dys-

regulation of alanine, aspartate, and glutamate metabolism—were present only in the circulation.

To resolve these metabolic dynamics more precisely, we applied c-means clustering to the 95 dysregulated metabolic peaks, identifying four trajectory patterns across disease stages (Figures 3D–3F; Figure S4B). Metabolites in cluster 1, including proline, phenylalanine, and lactic acid, increased sharply at the transition from benign lesions to AIS (Figure 3E), suggesting a potential metabolic inflection point during malignant transformation. In contrast, cluster 2 metabolites—such as 4-pyridoxic acid, glycocholic acid, and urea—shifted prominently at the transition to minimally invasive or invasive cancer (Figure 3G), consistent with metabolic rewiring required for tissue invasion. Several metabolites displayed monotonic changes across all stages, including sustained decreases in arginine-related metabolites and increases in palmitic acid (Figures 3H and 3I).

These observations are consistent with prior tissue-based studies showing declining arginine levels linked to reduced argininosuccinate synthase 1 expression and immune evasion, and they extend this biology into the circulation. By contrast, palmitic acid levels were increased in blood but not detected as altered in prior tumor tissue studies, pointing to a circulation-specific metabolic signature.

Development of a circulating metabolic diagnostic assay

Having delineated the circulating metabolic evolution of LUAD, we next sought to translate these findings into a clinically actionable diagnostic tool. Using the exploratory cohort, we developed the LUADMet assay—a diagnostic model built from circulating metabolites that reflect stage-specific metabolic dysregulation across the normal-benign-AIS-invasive continuum.

We applied a three-step development strategy encompassing feature discovery, feature optimization, and algorithm selection (Figure 4A). In the initial discovery stage, metabolites were filtered using three criteria ($p < 0.05$, $|\log_2(\text{fold change})| > 0.09$, and signal intensity > 300) to identify features with strong discriminative potential (Figure 4B). This yielded 54 candidate metabolic features. A neural network (NN) model trained on this full feature set achieved an area under the curve (AUC) of 0.900 (95% confidence interval [CI]: 0.883–0.916) for LUAD detection, with 83.19% sensitivity and 82.13% specificity (Figure 4C).

To enhance clinical applicability and reduce model complexity, we next refined the panel using a greedy feature optimization approach. Features were sequentially added to maximize diagnostic performance while minimizing noise (Figure 4D). This process identified 11 optimal features corresponding to 10 metabolites—lactic acid, proline, 4-pyridoxic acid, pipelicolic acid, ornithine, uric acid, glucose, phenylalanine, urea, and L-acetylcarnitine (Table S8). Each metabolite was selected based on consistent dysregulation observed across

(H) Representative metabolite showing stepwise increase from normal lung to invasive LUAD ($p < 0.001$, Kruskal-Wallis H test). The center line indicates the mean, the lower and upper hinges represent the 25th and 75th percentiles, and the whiskers extend from the 10th to the 90th percentile.

(I) Representative metabolite showing stepwise decrease from normal lung to invasive LUAD ($p < 0.05$, Kruskal-Wallis H test). The center line indicates the mean, the lower and upper hinges represent the 25th and 75th percentiles, and the whiskers extend from the 10th to the 90th percentile. TCA, tricarboxylic acid cycle.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Sample sizes for the normal, benign, AIS, and MIA/IAC groups are 84, 649, 214, and 500, respectively (e–i). See also Figure S4; Tables S5–S7.

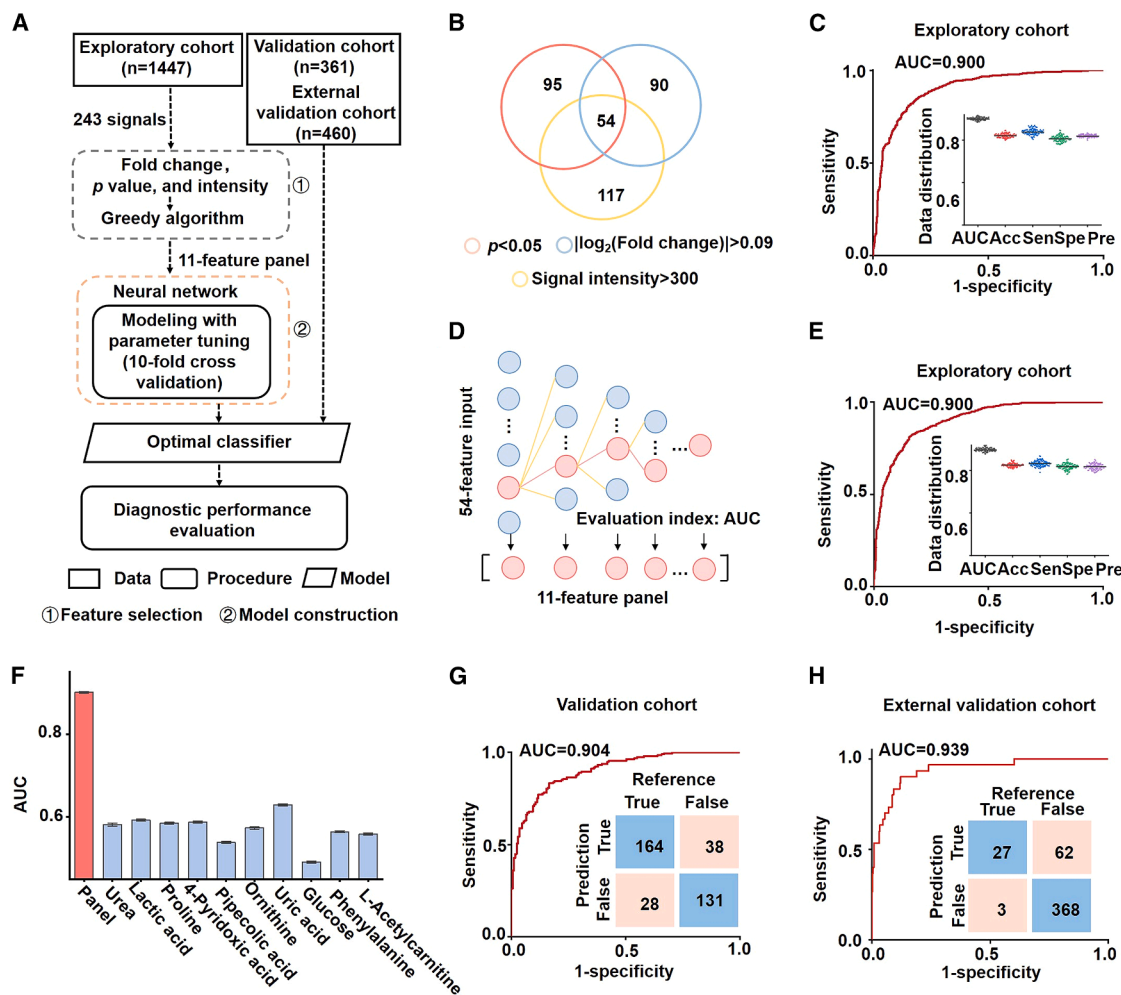


Figure 4. Development of the metabolic diagnostic assay

(A) Schematic overview of diagnostic model development and performance evaluation using retrospective cohorts.

(B) Selection of candidate metabolite features with diagnostic potential (two-sided t test).

(C) Receiver operating characteristic (ROC) curve of the neural network model distinguishing LUAD from non-LUAD controls in the exploratory cohort. Inset shows distributions of area under the curve (AUC), accuracy (Acc), sensitivity (Sen), specificity (Spe), and precision (Pre) ($n = 100$ bootstrap replicates).

(D) Greedy feature-selection strategy identifying an optimal 11-feature diagnostic panel.

(E) ROC curve of the neural network model based on the 11-feature panel in the exploratory cohort. Inset shows performance metric distributions. $n = 100$ bootstrap replicates.

(F) Comparative diagnostic performance of the combined panel versus individual metabolites. Data are shown as mean with 95% confidence intervals ($n = 100$ bootstrap replicates).

(G) ROC curve of the assay in the validation cohort (AUC = 0.904); inset shows the confusion matrix (accuracy = 81.72%).

(H) ROC curve of the assay in the external validation cohort (AUC = 0.939); inset shows the confusion matrix (accuracy = 85.87%). See also Figure S4, Tables S8–S14.

early and progressive disease stages, ensuring biological relevance to malignant transformation. Notably, based on the data available, we performed multivariable logistic regression analyses at the metabolite level in the exploratory cohort, which confirmed that the selected metabolites remain independently associated with LUAD status after adjustment for age and gender ($p < 0.05$, Table S9).

Pathway analysis based on selected 10 biomarkers revealed 5 significantly altered biological pathways (Table S10), among which arginine biosynthesis, arginine and proline metabolism,

and phenylalanine, tyrosine and tryptophan biosynthesis were the top three most prominently changed. The prominence of arginine-related pathways aligns with reported diminished circulating arginine availability in lung cancer patients, likely arising from tumor-driven suppression of arginine production as an adaptive response to counteract its T cell-mediated anti-tumor effects.^{37,38} Besides, the alteration of phenylalanine, tyrosine, and tryptophan biosynthesis pathway correlates with altered serum phenylalanine observed in non-small cell lung cancer patients. This metabolic shift is likely driven by reduced

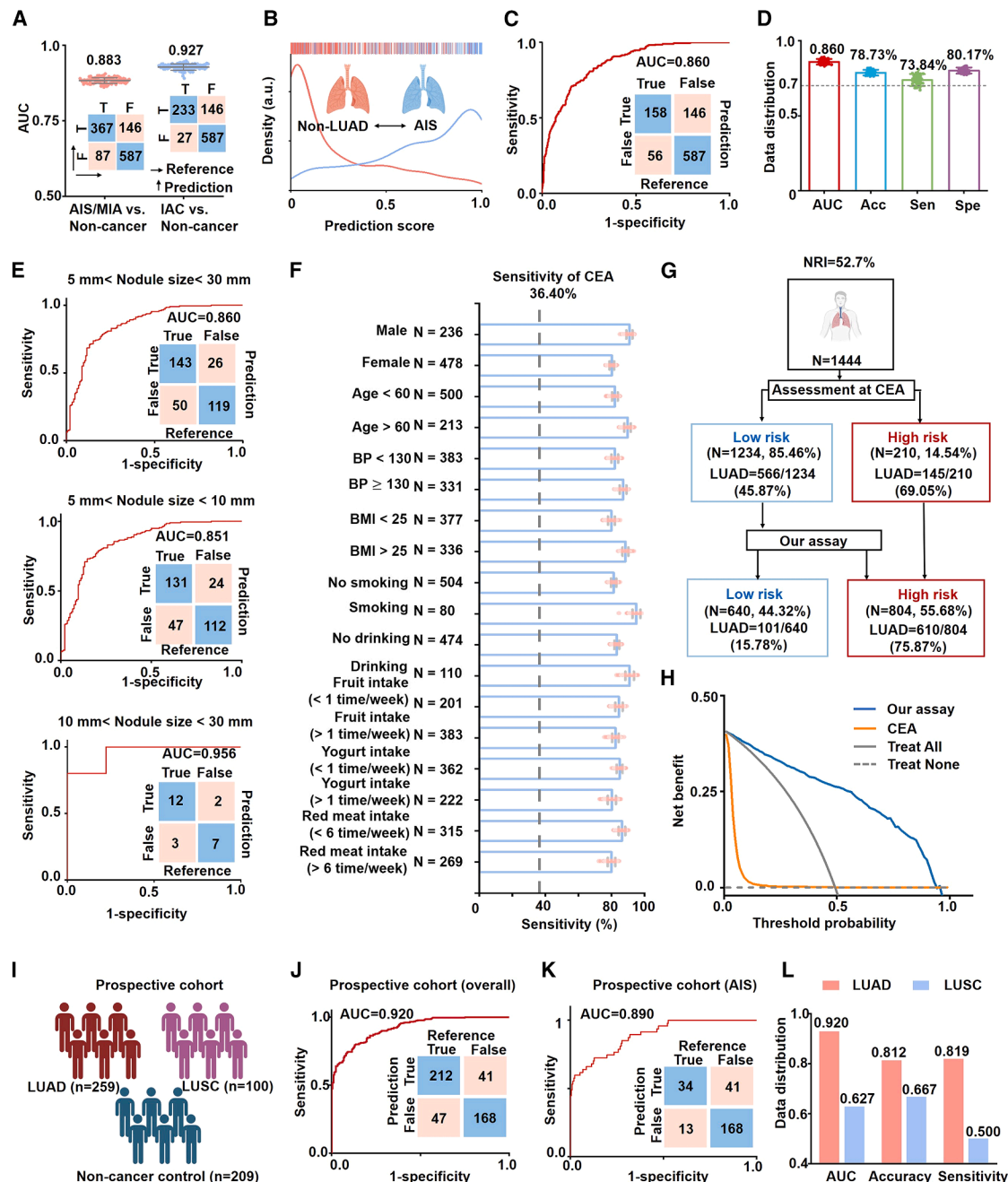


Figure 5. Assay performance in real-world clinical settings

(A) Distribution of AUC values for predicting AIS/MIA and IAC, with an AUC of 0.883 for early-stage LUAD. T, true; F, false. $n = 100$ bootstrap replicates.

(B) Distribution of prediction scores for AIS versus non-cancer controls.

(C) ROC curve for AIS detection (AUC = 0.860).

(D) Summary of five performance metrics for AIS detection. $n = 100$ bootstrap replicates. Data are represented as mean $\pm$ SEM.

(E) ROC curves for AIS detection stratified by nodule size: 5–30 mm (AUC = 0.860), 5–10 mm (AUC = 0.851), and 10–30 mm (AUC = 0.956).

(F) Sensitivity analysis across demographic and lifestyle subgroups, including gender, age, BMI, smoking status, alcohol use, and dietary habits. $n = 100$ bootstrap replicates.

(G) Schematic illustrating the incremental benefit of the assay in reclassifying initial triage decisions relative to CEA at first clinical contact.

(H) Decision curve analysis demonstrating potential net clinical benefit. Gray solid line (“Treat All”) denotes intervening on all patients regardless of any diagnostic results, while the gray dashed line (“Treat None”) denotes intervening on no patients regardless of any diagnostic results.

(I) Composition of the prospective cohort.

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phenylalanine hydroxylase activity and tyrosine-mediated immune dysfunction linked to malignant progression.^{39,40}

To investigate the biological origin of the candidate biomarkers, we analyzed paired serum samples from 30 patients with invasive LUAD, collected before and after curative surgical resection (Table S11). Using NPELDI-MS, we measured the levels of the ten selected biomarkers in these samples (Figure S4C; Table S12). Following surgery, the levels of urea, ornithine, and L-acetylcarnitine returned to normal (non-cancer) status, suggesting that these metabolites are likely tumor derived and may directly reflect the presence or metabolic activity of cancer cells. In contrast, the levels of lactic acid, proline, 4-pyridoxic acid, pipercolic acid, uric acid, glucose, and phenylalanine remained unaltered after tumor removal. This persistence indicates that these metabolites may represent a systemic, persistent host-response metabolic reprogramming induced by the cancer, which continues even after the primary tumor has been resected.

Using this 10-biomarker panel, we compared eight machine learning algorithms—neural network (NN), random forest, ridge regression, naive bayes, support vector machine, adaptive boosting, lasso regression, and gradient boosting—to construct the final diagnostic model. The NN-based LUADMet assay performed best, achieving an AUC of 0.900 (95% CI: 0.885–0.916), with 84.03% sensitivity and 80.00% specificity in the exploratory cohort (Figure 4E; Table S13). Performance was statistically indistinguishable from the original 54-feature model ($p > 0.05$, DeLong test) and demonstrated excellent stability across evaluation metrics (Figures 4C and 4E; Table S14). As expected, single-metabolite classifiers performed markedly worse (AUC range: 0.505–0.630; Figure 4F).

To assess reproducibility and generalizability, the LUADMet assay was evaluated in two independent retrospective cohorts. The validation cohort, with balanced demographic characteristics ($p > 0.05$), provided an ideal benchmark for technical performance, yielding an AUC of 0.904 (95% CI: 0.874–0.934) (Figure 4G). The external validation cohort, characterized by marked age imbalance that more closely reflect real-world heterogeneity under certain screening scenarios, produced an AUC of 0.939 (95% CI: 0.898–0.980) (Figure 4H). These values were consistent with performance in the exploratory cohort, collectively demonstrating robustness across population settings. Notably, to evaluate LUADMet performance independent of age, we additionally performed a covariate-adjusted analysis in the external validation cohort, incorporating the LUADMet's prediction score and age as covariates, following established methods.^{41,42} The age-adjusted diagnostic AUC of LUADMet was 0.900 (Figure S4D), showing no significant change from the unadjusted ones ($p = 0.426$, DeLong test). The maintained AUCs demonstrated that our assay is robust to age effects.

Together, these findings support LUADMet as a biologically grounded approach that may aid in detecting early-stage LUAD across diverse patient groups.

Assay evaluation for real-world clinical application

To assess the translational relevance of the LUADMet assay, we evaluated its performance in two major clinical challenges: early-stage detection and classification across diverse patient populations. For early-stage disease, the assay achieved an AUC of 0.883 (95% CI: 0.881–0.885) for distinguishing AIS or MIA from controls (Figure 5A). When restricted to pure AIS, performance remained robust, with an AUC of 0.860 (95% CI: 0.857–0.862), 73.83% sensitivity, and 80.08% specificity (Figures 5B–5D).

Clinically, indeterminate pulmonary nodules measuring 5–30 mm—particularly those without definitive benign features—represent one of the most challenging diagnostic scenarios in early lung cancer.⁴³ Current CT-based guidelines often require 12–24 months of radiographic follow-up before malignancy can be confidently excluded or confirmed.⁴⁴ In this subgroup, LUADMet achieved an AUC of 0.860 (95% CI: 0.821–0.899; false-positive rate 17.9%) for identifying malignant nodules (Figure 5E) and an AUC of 0.851 (95% CI: 0.812–0.894) for nodules <10 mm, demonstrating that metabolic alterations are detectable prior to overt morphological progression.

We next evaluated LUADMet across clinically relevant subpopulations. The assay maintained >80% sensitivity at 80% specificity across strata defined by age, gender, blood pressure, smoking history, alcohol consumption, and dietary patterns (fruit, yogurt, and red meat intake) (Figure 5F). By comparison, CEA demonstrated only 36.40% sensitivity at the same specificity threshold.

To further assess clinical utility, we compared LUADMet against CEA using net reclassification improvement (NRI), a widely applied metric for evaluating added value in risk stratification.⁴⁵ Using CEA as the baseline model, 85.46% of individuals were classified as low risk (negative predictive value [NPV]: 54.13%, 668/1234) and 14.54% as high risk (positive predictive value [PPV]: 69.05%, 145/210). Integration of LUADMet reclassified 41.14% additional high-risk individuals, improved NPV to 84.22% and PPV to 75.87%, and increased sensitivity by 63.99%, yielding an NRI of 52.66% (Figure 5G). Decision curve analysis further supported a superior net clinical benefit (Figure 5H).

Because LUADMet has potential relevance for screening, we additionally evaluated its performance in a prospective cohort of 568 consecutively enrolled individuals (259 LUAD; 100 lung squamous cell carcinoma [LUSC]; 209 non-cancer; Figure 5I; Table S15). The clinical characteristics of LUAD patients mirrored those reported in large Chinese screening cohorts,^{46–48} with 88.4% presenting with stage 0–I disease, 8.5% with stage II, and 3.1% with stage III–IV disease. To reflect real-world screening environments where multiple cancer types may be encountered, LUSC cases were included to assess assay specificity. LUADMet distinguished LUAD from non-cancer individuals with an AUC of 0.920 (95% CI: 0.896–0.943; sensitivity 81.9%, specificity 80.4%) and maintained strong performance in AIS (AUC 0.890, 95% CI: 0.844–0.936; Figures 5J and 5K). By contrast, the assay showed limited ability to discriminate

(J) ROC curve for assay performance in the prospective cohort (AUC = 0.920); inset shows the confusion matrix (accuracy = 81.20%).

(K) ROC curve for AIS detection in the prospective cohort (AUC = 0.890); inset shows the confusion matrix (accuracy = 78.91%).

(L) Comparison of assay performance between LUAD and lung squamous cell carcinoma (LUSC). CEA, carcinoembryonic antigen. See also Figure S4, Table S15.

LUSC from non-cancer cases (AUC 0.627, 95% CI: 0.566–0.688; [Figure 5L](#)), consistent with preferential performance in LUAD. We also added age-adjusted analysis in the prospective cohort. The diagnostic AUC remained comparable before and after adjustment (0.920 v.s. 0.903, $p = 0.386$, DeLong test, [Figure S4E](#)), further supporting the robustness of the assay to age effects within the studied population.

We further performed the estimation under realistic prevalence assumptions to better evaluate the performance of the LUADMet assay in screening based on parameters reported in the TALENT trial (see details in [STAR Methods](#)).⁴⁹ As a result, in the LDCT-positive high-risk population, the estimated PPV and NPV for LUADMet are 40.6% and 96.8%, respectively. This PPV represents an increase of 26.4 percentage points over the positive predictive value of LDCT alone (14.0%). In a general screening scenario (approximated by the baseline prevalence of 2.6%), the estimated PPV and NPV are 10.1% and 99.5%, respectively. These results support the clinical utility of deploying LUADMet after a positive LDCT result, where it can effectively further stratify high-risk individuals and help identify those with a high probability of malignancy.

Considering the deployability of LUADMet as a clinical assay in a single-sample or small-batch clinical laboratory setting, we designed a random forest-based QC signal correction (QC-RFSC) workflow specifically for prospective use (see details in [STAR Methods](#)). The workflow only requires the analysis of a standardized QC sample alongside the patient sample to perform correction. Notably, the performance of the corrected LUADMet model is comparable to that of the non-QC corrected model (AUC of 0.887 vs. 0.900, [Figure S4F](#)) in the exploratory cohort, demonstrating the utility of this fixed procedure. This approach is practical for clinical MS, making the LUADMet assay deployable across different sites, time periods, and instrument batches.

DISCUSSION

This study defines a large, patient-derived circulating metabolomic resource that captures metabolic remodeling across the continuum of LUAD development. By reconstructing serum metabolic trajectories spanning normal lungs, benign lesions, AIS, MIA, and IAC, we show that reproducible metabolic alterations are detectable at pre-invasive stages. These observations are consistent with tissue-based metabolomic studies reporting early perturbations in amino acid metabolism, including arginine and proline pathways, as well as progressive rewiring of glycolytic and nucleotide biosynthetic programs during LUAD evolution.^{34,50} Our findings extend these insights to the circulation, a clinically accessible compartment in which early carcinogenesis-associated metabolic changes remain comparatively underexplored.

The scale and design of the cohort were critical for resolving stage-associated metabolic patterns against inter-individual variability. With 2,836 participants across integrated retrospective and prospective cohorts, this study exceeds the size of most prior LUAD serum metabolomics investigations, which have often been limited by modest sample sizes, incomplete representation of early-stage disease, or the absence of normal

controls.^{34,50,51} The ultrafast NPELDI-MS workflow enabled high-throughput analysis of 2,268 serum samples under uniform analytical conditions, helping to reduce technical variability that commonly constrains chromatography-based metabolomic approaches.^{52–54} Importantly, inclusion of individuals with normal lungs—rarely feasible in tissue-based studies—provided a reference framework for distinguishing physiological baseline variation from disease-associated metabolic alterations. Within this context, our dataset recapitulated established metabolic pathways implicated in LUAD progression, such as glycine, serine, and threonine metabolism and pyrimidine biosynthesis,³⁴ while also highlighting less characterized pathways, including galactose metabolism, and dynamic metabolite trajectories that have not been systematically examined in prior tissue-based studies.

Regarding the within-person reproducibility of metabolic signatures, recent high-frequency sampling studies indicate that while blood metabolic profiles do fluctuate, many variations follow reproducible circadian rhythms. Specifically, >300 metabolites, including amino acids and carbohydrates, acetylcarnitine occurred in our biomarker panel, show significant diurnal patterns in 7 days.⁵⁵ This reproducible fluctuation suggests that measured levels of blood metabolites can be stable if blood sampling is performed at a fixed time. Besides, previous reports also demonstrated that under standardized fasting conditions, blood metabolites exhibit good short-term reproducibility (median intra-class correlation coefficient [ICC] = 0.67).⁵⁶ Together, these evidences indicate that standardizing sampling conditions (e.g., fixed time, fasting state) can effectively capture a stable metabolic baseline, therefore promising the within-person reproducibility of metabolic signatures. Exactly, our study was designed according to this principle. All serum samples were collected under clinical standardized fasting conditions between 6 and 9 am. Therefore, the identified LUADMet signature reflects a metabolic state under controlled homeostasis, providing a foundation for its reliable longitudinal application.

From a clinical perspective, the observation that circulating metabolic alterations precede overt structural invasion has potential implications for early detection strategies. LDCT screening is associated with high false-positive results, frequently exceeding 90%, potentially resulting in prolonged surveillance and unnecessary diagnostic procedures.^{17,57} Existing circulating biomarkers,⁵⁸ including protein-based markers such as CEA, show limited sensitivity for early-stage disease.⁵⁹ Although the combination of conventional protein biomarkers (e.g., the INTEGRAL panel) has been shown to improve diagnostic performance, especially in never-smoking population, their performance in detecting early-stage disease remains limited, with reported AUC values still below 0.7.^{16,60} Emerging assays based on circulating tumor DNA or small RNAs often demonstrate variable or modest sensitivity in rigorously validated cohorts, particularly for pre-invasive lesions.⁶¹ Metabolomics profiles the downstream functional outputs of cellular activity. The resulting systemic shifts in metabolite levels may be amplified relative to upstream genomic or proteomic changes, potentially offering a more sensitive window into early pathogenesis.^{50,62,63} Prior metabolomic studies have suggested potential utility for early lung cancer detection, reporting AUC values

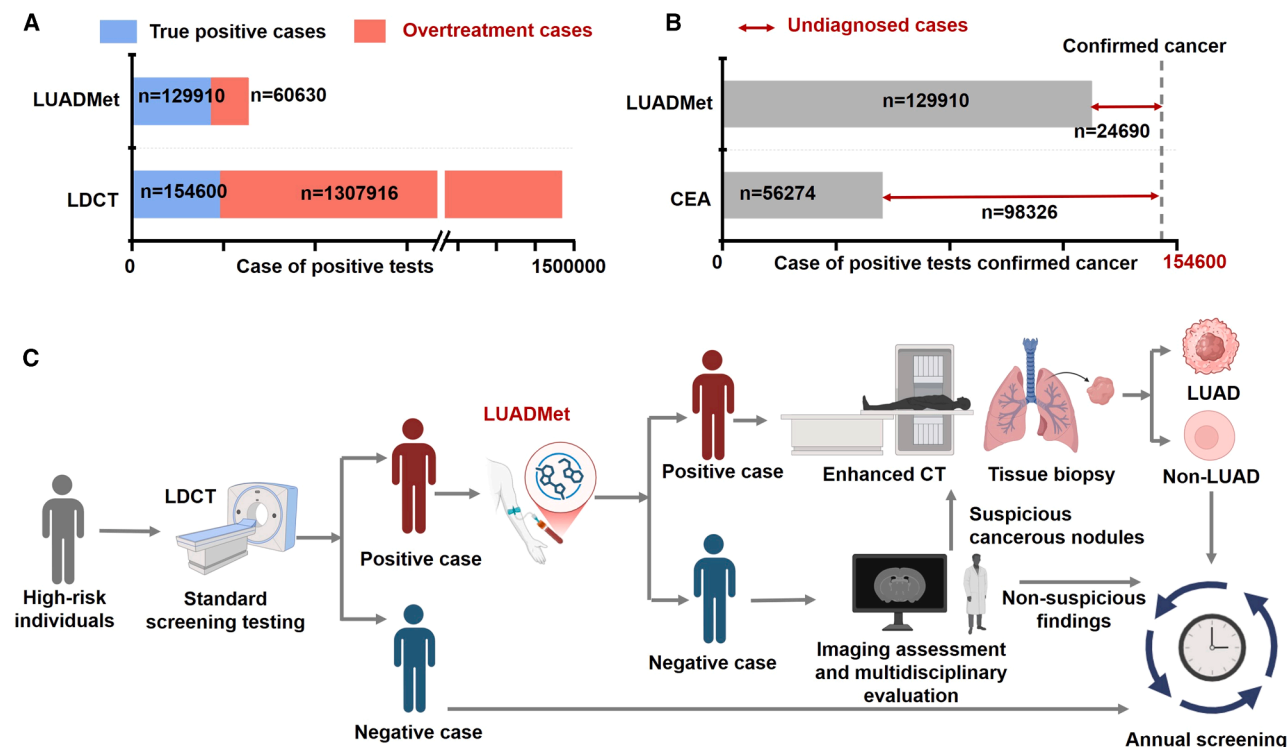


Figure 6. Proposed clinical implementation of LUADMet for LUAD screening

(A) Estimated reduction in overtreatment using LUADMet compared with standard LDCT screening in a simulated Chinese screening population.

(B) Estimated reduction in undiagnosed cases using LUADMet compared with CEA-based testing.

(C) Proposed screening framework integrating LUADMet as a complementary tool within clinical workflows. LUADMet may be applied following a positive LDCT result to refine diagnostic decision-making.

-ranging from approximately 0.80 to 0.91 for early-stage LUAD.^{50,64,65} However, these studies largely focused on MIA and IAC stages and generally lacked sufficient resolution for reliable detection of AIS. Against this background, LUADMet—derived from stage-informed metabolic trajectories—demonstrated reproducible performance across multiple clinically relevant settings, including AIS and indeterminate pulmonary nodules. Specifically, the LUADMet assay achieved a sensitivity of 50% at 98% specificity for stage I LUAD. Importantly, it retained a sensitivity of 30% for adenocarcinoma *in situ* (stage 0) at the same high specificity. More importantly, our assay demonstrates competitive performance within the predominantly never-smoking population studied, which is a kind of cohort that has been relatively underexplored in prior biomarker research.

The stability of LUADMet performance across demographic, clinical, and lifestyle subgroups further supports the robustness of early metabolic alterations observed in this cohort. This contrasts with some protein- or nucleic acid-based biomarkers, whose performance can be influenced by tumor burden, shedding efficiency, or patient heterogeneity. Critically, the assay's cost effectiveness (<\$1/sample) and rapid turnaround (15 min) further support scalability, particularly in resource-limited settings. In contrast, the average metabolic analysis time per sample using a conventional LC-MS platform is estimated to be over 5 h, mainly including protein removal (>2 h), analyte concentration (3–6 h), and chromatographic separation and ionization (15–30 min). This

duration is obviously longer than that of our assay, highlighting the speed advantage of our assay in scalable detection.

Based on the inherent advantages of the LUADMet assay, we integrated the model into existing clinical workflows and assessed clinicians' acceptance of diagnostic tools. When modeled for population screening in China (Figures 6A and 6B), where ~154,600 LUAD cases exist among 55 million high-risk residents according to 2022 statistics, our assay potentially reduces overtreatment by >58% compared to LDCT (preventing >1,247,000 false positive cases and saving >\$374 million in unnecessary costs) and reduces missed diagnosed rate by 47% v.s. CEA (avoiding ~73,636 undiagnosed cases).⁶⁶ Importantly, the practical deployment of our assay, especially in resource-limited settings, could be further enhanced by adopting simplified sample storage carriers, such as dried blood or serum spots. These tools minimize logistical demands and costs by reducing the need for specialized tubes and cold-chain transport, directly addressing key barriers to population-based lung disease screening and complementing the assay's inherent advantages of low cost and rapid analysis.

Rather than replacing existing screening modalities, LUADMet is best viewed as a complementary approach that may enhance clinical decision-making (Figure 6C), especially in a specific Chinese, predominantly female, never-smoking population. Potential use scenarios include post-LDCT risk stratification of indeterminate nodules or earlier deployment to identify individuals who

may benefit most from imaging-based screening. Such integrative strategies align with emerging paradigms that combine molecular and radiologic information to improve screening accuracy while minimizing unnecessary interventions. Notably, LUADMet is optimized for the detection of LUAD and exhibits low expected sensitivity for LUSC. Accordingly, a negative LUADMet result indicates a low probability of adenocarcinoma but does not rule out LUSC or other subtypes. Therefore, the management of patients with a negative test result should continue to be guided by comprehensive imaging assessment and multidisciplinary evaluation. Nodules displaying radiological features suggestive of LUSC should be referred for further diagnostic or therapeutic evaluation, whereas a negative result at this stage can be considered reassuring.

In summary, this study provides (1) validation of a metabolic profiling platform suitable for large-scale serum analysis, (2) a high-resolution circulating metabolic atlas spanning LUAD progression, and (3) a clinically scalable, non-invasive assay informed by stage-specific metabolic trajectories. Together, this resource supports the integration of metabolomics into next-generation lung cancer screening strategies and provides a reference framework for future studies of early carcinogenesis.

Limitations of the study

Several limitations should be acknowledged. First, the metabolic atlas and LUADMet classifier were developed and validated in single-center cohorts derived exclusively from a Chinese, predominantly female, never-smoking population. Therefore, further evaluation in geographically and ethnically diverse, smoking-enriched multi-center cohorts is an essential next step to establish broader applicability. Second, although our case-control design provides strong initial evidence for diagnostic utility, prospective interventional studies embedded within real-world screening programs will be needed to determine how LUADMet performs in routine clinical workflows and whether its use improves downstream patient outcomes. Moreover, future prospective validation with comprehensive data collection (e.g., repeated sampling at fixed intervals under the same conditions) is required to fully adjust for other covariates (e.g., smoking history, BMI, and dietary variables) at the metabolite level to support assay robustness. Finally, future studies should also investigate the high-resolution circulating metabolic atlas of LUSC progression. This would enable the development of a LUSC-specific metabolic assay, which could then be integrated with our LUADMet to enhance the current screening workflow.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lin Huang (linhuang@shsmu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This study has not created any original computer code, algorithms, or computational models.

- De-identified clinical and metabolomic data supporting the findings of this study are included in the manuscript and supplementary data. Requests for additional de-identified clinical data will be considered subject to legal, ethical, and institutional approvals, as guided by the lead clinicians (Dr. Tianxiang Chen, Shanghai Chest Hospital). The standardized metabolomics data have been deposited to the NIH Common Fund's National Metabolomics Data Repository (NMDR) Website, the Metabolomics Workbench: <https://www.metabolomicsworkbench.org>, where it has been assigned study ID: ST004975 (<https://doi.org/10.21228/M8TP1M>), as listed in the [key resources table](#).
- Any additional information required to reanalyze the data reported in this work paper is available from the [lead contact](#) upon request.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant nos. 82422042, 824B2059, and 82372148), National Key R&D Program of China (grant no. 2025YFE0206800), the Medical-Engineering Joint Funds of Shanghai Jiao Tong University (grant nos. YG2023ZD08 and YG2024ZD07), and the Sichuan Science and Technology Program (grant no. 2024YFHZ0176).

AUTHOR CONTRIBUTIONS

L.H., D.T., K.Q., and J.W. conceived and designed the study. S.Y., Y.Q., and T.C. performed the experiments and drafted the initial manuscript. T.C., W.Y., Y.Z., and H.M. contributed to biospecimen collection and clinical coordination. S.Y., A.Y., C.D., Y.L., K.M., and W.L. conducted the mass spectrometry experiments, performed data analyses, and prepared the figures and manuscript. Y.Q., F.M., J.W., J.Z., Y.H., X. Xu, and X. Xie assisted with mass spectrometry experiments and data processing. R.K. assisted with manuscript editing and data interpretation. All authors contributed to data analysis and interpretation, participated in critical discussions, and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare competing interests related to patents filed on the technology described herein and its applications in biosample detection.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplementary data related to this article can be found online at <https://doi.org/10.1016/j.xcrm.2026.102991>.

Received: March 9, 2026

Revised: June 5, 2026

Accepted: July 20, 2026

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human serum samples	Department of Surgical Oncology, Shanghai Lung Cancer Center, Shanghai Chest Hospital	NA
Chemicals, peptides, and recombinant proteins		
Proline	Sigma-Aldrich	Cat# P0380
Alanine	Sigma-Aldrich	Cat# A7627
Methionine	Sigma-Aldrich	Cat# S167794
Lactic acid	Sigma-Aldrich	Cat# L1750
Malic acid	Sigma-Aldrich	Cat# 02288
Succinic acid	Sigma-Aldrich	Cat# 398055
Inosine	Sigma-Aldrich	Cat# I4125
Glycine	Sigma-Aldrich	Cat# G7126
Valine	Sigma-Aldrich	Cat# V0500
Glucose	Sigma-Aldrich	Cat# 49139
Sucrose	Sigma-Aldrich	Cat# 16104
Ferric chloride hexahydrate	Sinopharm Chemical Reagent	Cat# 10025-77-1
Anhydrous sodium acetate	Sinopharm Chemical Reagent	Cat# 127-09-3
Trifluoroacetic acid	Sigma-Aldrich	Cat# T6508
Bovine serum albumin	Sigma-Aldrich	Cat# A9418
Arginine	Shanghai Aladdin Reagent	Cat# A137768
Phenylalanine	Shanghai Aladdin Reagent	Cat# P103489
Histidine	Shanghai Aladdin Reagent	Cat# H111055
Pyruvic acid	Shanghai Aladdin Reagent	Cat# P104138
Thymine	Shanghai Aladdin Reagent	Cat# T108391
Glycodeoxycholic acid	Shanghai Aladdin Reagent	Cat# G1495713
Linoleic acid	Shanghai Aladdin Reagent	Cat# L100441
Fructose	Shanghai Aladdin Reagent	Cat# F304579
Deposited data		
LDI-MS based metabolomics	This paper	NMDR: ST004975, Project: https://doi.org/10.21228/M8TP1M
Software and algorithms		
R	R Foundation	https://www.r-project.org/ ; version 4.4.0; RRID: SCR_001905
Python	Python Software Foundation	https://www.python.org/ ; version 3.7.0;
MetaboAnalyst	Xia Lab	https://www.metaboanalyst.ca/ ; version 6.0; RRID: SCR_015539
SPSS	IBM	https://www.ibmsspss.cn/ ; version 24.0;
BioRender	BioRender	https://www.biorender.com/ ; RRID: SCR_018361
Progenesis QI	Waters	https://www.waters.com/nextgen/cn/zh/products/informatics-and-software/mass-spectrometry-software/progenesis-qi-software.html ; version 2.0;
HMDB	HMDB Foundation	https://www.hmdb.ca ; RRID:SCR_007712
DataAnalysis software	Bruker	version 5.0;
FlexAnalysis software	Bruker	version 3.4;

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Clinical cohort and sample collection

Participants were enrolled at Shanghai Chest Hospital and comprised three retrospective Chinese cohorts ($n = 2,268$) and one prospective Chinese cohort ($n = 568$). The retrospective cohorts included an exploratory cohort (714 LUAD cases and 733 non-LUAD controls), a validation cohort (192 cases and 169 controls), and an external validation cohort (30 cases and 430 controls), with enrollment occurring between 2019 and 2021. The exploratory and validation cohorts were designed to support diagnostic assay development and evaluation using an approximate 4:1 sample allocation, while ensuring balanced age and gender distributions between LUAD and non-LUAD groups to minimize demographic confounding (χ^2 test for gender; two-sided t test for age; $p > 0.05$). In contrast, the external validation cohort intentionally incorporated broader demographic variability to better reflect real-world clinical heterogeneity and to assess assay generalizability.

The prospective cohort, enrolled beginning in 2022, consisted of 259 newly diagnosed LUAD cases and 309 control individuals. To further evaluate assay specificity, 100 cases of lung squamous cell carcinoma (LUSC) were included within the control group. All participants were randomly selected at their initial clinical presentation and had not received any anticancer treatment prior to sample collection or surgical intervention.

LUAD cases were excluded if they met any of the following criteria: (1) diagnosis of a non-LUAD malignancy; (2) absence of available pathological specimens; (3) inadequate or turbid serum samples; (4) history of other malignant tumors or severe systemic diseases; or (5) incomplete follow-up information. All included LUAD cases were confirmed by histopathological examination. In a minority of cases where discrepancies occurred between the clinical report and the tissue diagnosis, the definitive diagnosis was established through a formal multidisciplinary team (MDT) review. In detail, radiological diagnoses were recorded through an independent, blinded review of original reports and key imaging features, conducted by two specialized radiologists (with 11 and 14 years of experience in cancer imaging, respectively). This impression was subsequently compared with the histopathological diagnosis by two thoracic clinicians (each with >10 years of clinical diagnosis experience). Any discordant cases were resolved through MDT arbitration, which established the final “ground-truth” label for all samples.

Peripheral blood samples were collected after overnight fasting using serum separator tubes without anticoagulants. Samples were allowed to clot at room temperature for 40 min and were then centrifuged at $5,100 \times g$ for 10 min at 4°C . Serum supernatants were aliquoted, immediately snap-frozen, and stored at -80°C until analysis. A pooled quality control (QC) sample was generated by combining 20 μL aliquots from 100 LUAD serum samples and 100 non-cancer control samples and was used to monitor analytical stability across batches.

Baseline demographic characteristics, clinical laboratory parameters, lifestyle factors, and dietary information were obtained from electronic medical records and structured follow-up assessments. Final cohort sizes met minimum requirements established by an *a priori* power analysis. Adjustment analyses for gender and age were conducted to ensure the robustness of our diagnostic model against potential confounding effects. The study protocol and all sample collection procedures were approved by the Institutional Ethics Committee of Shanghai Chest Hospital (approval number: KS22025), and written informed consent was obtained from all participants.

Randomization and blinding

Participant inclusion within each cohort was performed using a random selection strategy at the time of initial clinical presentation to minimize selection bias. For retrospective cohorts, eligible cases and non-cancer controls were randomly sampled from institutional databases according to predefined inclusion and exclusion criteria. For the prospective cohort, consecutive eligible participants were enrolled and assigned study identifiers in a randomized order prior to downstream processing.

All serum samples were de-identified and assigned randomized codes before metabolomic analysis. Investigators performing sample processing, mass spectrometry acquisition, and data preprocessing were blinded to clinical diagnosis, pathological stage, and cohort assignment. Diagnostic modeling and statistical analyses were performed using coded datasets, and clinical labels were revealed only after completion of primary analyses.

METHOD DETAILS

Material characterization

Digital images of the mass spectrometer-coupled microarray chip were acquired using an iPhone 12 smartphone (Apple Inc., USA) equipped with a 12-megapixel wide-angle camera. Focus and exposure were manually locked on the chip surface under consistent ambient lighting, and images were saved in the highest-resolution format. Scanning electron microscopy (SEM) was performed using an S-4800 microscope (Hitachi Ltd., Japan). For SEM analysis, 10 μL of material suspension was deposited onto aluminum foil and air-dried. Micrographs were acquired at an accelerating voltage of 5 kV at magnifications of $100,000\times$ (scale bar, 100 nm) and $20,000\times$ (scale bar, 400 nm).

Metabolic detection using NPELDI-MS

Metabolic detection in this study comprised evaluation of the analytical performance of NPELDI-MS and application of this platform for serum metabolic fingerprinting.

Ferric nanoparticles, used as the matrix for NPELDI-MS, were synthesized via a solvothermal method as previously described.⁵¹ Briefly, ferric chloride hexahydrate and trisodium citrate dihydrate were dissolved in ethylene glycol under continuous stirring to generate a homogeneous solution. An aqueous solution of anhydrous sodium acetate was then added, and the resulting mixture was transferred to a sealed reactor and heated at 200°C for 10 h. After cooling, the reaction products were collected by centrifugation, washed repeatedly with deionized water, and dried under ambient conditions. For mass spectrometry analysis, ferric nanoparticles were resuspended in deionized water to generate a matrix slurry at a final concentration of 1 mg mL⁻¹.

The analytical performance of the NPELDI-MS platform was assessed in terms of sensitivity, specificity, and reproducibility. Sensitivity was evaluated using serial dilutions of metabolite standards. For each analysis, 1.5 μ L of metabolite solution was mixed with an equal volume of ferric nanoparticle matrix slurry, deposited onto the target plate, and air-dried at room temperature prior to MS acquisition. Limits of detection were determined from the resulting mass spectra. Specificity was evaluated by assessing the ability of the ferric nanoparticle matrix to selectively ionize low-molecular-weight metabolites in the presence of high-abundance proteins. A standard mixture containing small-molecule metabolites (1 mg mL⁻¹) and bovine serum albumin (BSA; 5 mg mL⁻¹) was analyzed, and mass spectra were acquired across two mass ranges (m/z 100–600 and m/z 30,000–200,000) to characterize ionization selectivity. Reproducibility was assessed by repeated analysis of a metabolite standard solution (1 mg mL⁻¹) across multiple discrete spots on the same target plate. Intra-plate signal stability was quantified as the coefficient of variation (CV) of signal intensities across replicates.

For comparison, identical analytical evaluations were performed using conventional organic matrices, including α -cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB). These matrices were prepared at a concentration of 10 mg mL⁻¹ in TA30 solvent (acetonitrile/water containing 0.1% trifluoroacetic acid, 7:3, v/v).

For serum metabolic fingerprinting, all serum samples were diluted 10-fold with deionized water. An aliquot (1.5 μ L) of diluted serum was mixed with an equal volume of ferric nanoparticle matrix slurry, and 1.5 μ L of the resulting mixture was deposited onto the target plate for MS analysis.

All mass spectrometry data were acquired in positive ion mode using a Bruker Autoflex Speed TOF/TOF mass spectrometer (Bruker, Bremen, Germany) operated in reflectron mode. The instrument was equipped with a Nd:YAG laser (355 nm) operated at a repetition rate of 1,000 Hz, with an acceleration voltage of 20 kV.

For assessment and correction details in run-order signal drift, the MS instrument software performed real-time monitoring of key parameters during data acquisition in the QC samples, including the mass accuracy and signal intensity of known metabolites (e.g., glucose, valine, and alanine). To ensure consistent mass accuracy, a linear model-based correction was applied automatically to the mass axis whenever a mass deviation exceeding 50 ppm was detected in the QC spectra. For the criteria used to exclude runs, we implemented a QC signal-based halt procedure. If the signal intensity for key metabolites in a QC sample fell below a pre-defined threshold (10,000 a.u.), the acquisition run was automatically paused with an alert. The instrument was then inspected and re-tuned. Data collection resumed only after subsequent QC samples confirmed the restoration of stable metabolite signals.

MS data analysis

Following data acquisition, mass spectra were exported using FlexAnalysis Batch Process software (version 3.4; Bruker, Germany). The full spectral data, including mass-to-charge (m/z) values and corresponding signal intensities, were retained without thresholding for downstream preprocessing and analysis.

Serum metabolic fingerprint data were processed using a custom computational pipeline implemented in Python (version 3.7).⁶⁷ For each sample, spectra from five technical replicates were collected and aligned to generate a unified representation. Spectral alignment was performed by cubic spline interpolation using the `scipy.interpolate.splrep` function to generate spline representations and the `scipy.interpolate.splev` function to evaluate spectra on a common m/z grid. Intensity values were subsequently averaged across replicates (axis = 0) to improve signal stability.

The averaged spectra were subjected to Gaussian smoothing using `scipy.ndimage.Gaussian_filter` with a sigma value of 1 to reduce high-frequency noise while preserving peak shape. The rationale for spectral-level averaging is in 1) improving the signal-to-noise ratio (S/N) of the composite spectrum and 2) minimizing the variance introduced by the stochastic nature of ion detection in any single MS scan.^{68,69} In this way, it can improve the sensitivity and reproducibility of peak detection and thus increase the number and signal stability of detectable low-abundance features. Baseline correction was then applied using a morphological filtering approach implemented in the OpenMS toolkit (`pyopenms.MorphologicalFilter`) to remove low-frequency background signals attributable to instrumental drift or matrix-related effects.

Peak detection was performed on baseline-corrected spectra by identifying local maxima using `scipy.signal.find_peaks`. A consensus peak list was then constructed by aggregating m/z values from all detected peaks across samples. Individual sample peak lists were aligned to this consensus using the `pyopenms.SpectrumAlignment` algorithm, which employs a dynamic programming approach to optimize m/z matching. For peaks absent in individual samples, missing values were imputed using intensity values extracted from the corresponding m/z positions in the original baseline-corrected spectra, minimizing information loss while preserving sample-specific signal structure.

Finally, the processed data were corrected using the `combat.pycombat` algorithm and subsequently exported to a CSV file for downstream statistical analyses and machine learning modeling.

Parameter selection and justification

Parameter values used for spectral alignment, smoothing, peak detection, and filtering were selected based on empirical optimization using representative subsets of the data and prior experience with NPELDI-MS-based serum metabolomics. The Gaussian smoothing parameter ($\sigma = 1$) was chosen to reduce high-frequency noise while preserving peak shape and resolution. Peak detection thresholds (minimum prominence of 50 intensity units, minimum width of five data points, and minimum inter-peak distance of 15 data points) were selected to balance sensitivity and specificity and to minimize detection of noise-derived or overlapping features. A S/N ratio cutoff greater than 3 was applied to retain robust spectral signals commonly used in mass spectrometry analyses. ComBat correction was employed to correct feature scales across samples and any potential run-order signal intensity drift, thereby further enhancing the robustness of the downstream metabolomic analysis. This facilitates unbiased statistical comparison and machine learning modeling without imposing distributional assumptions.

Biomarker identification

Putative metabolites corresponding to characteristic MS features were annotated using a combination of two orthogonal analytical platforms and a curated reference database: Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS; Solarix 7.0 T, Bruker), liquid chromatography–mass spectrometry (LC-MS; Vanquish UHPLC coupled to a Q Exactive Plus mass spectrometer, Thermo Fisher Scientific), and the Human Metabolome Database (HMDB; <https://hmdb.ca/>).⁷⁰ Only metabolites independently identified by both FT-ICR MS and LC-MS were retained for downstream analyses and considered high-confidence annotations.

For FT-ICR MS analysis, sample preparation and matrix application followed the same procedures described for NPELDI-MS. Spectra were acquired with a laser spot diameter of approximately 50 μm . Data collection was performed in positive ion mode with detector plate voltage and offset set to 210 V and 80 V, respectively. Metabolite annotation was conducted using DataAnalysis software (version 5.0; Bruker) with a signal-to-noise threshold greater than 3 and a mass accuracy tolerance of less than 15 ppm relative to reference masses in HMDB.

For LC-MS analysis, a pooled serum sample was generated by combining equal volumes (10 μL each) from 24 individual serum samples, including 12 randomly selected LUAD cases and 12 non-cancer controls. A 100 μL aliquot of the pooled serum was subjected to protein precipitation by addition of 400 μL of ice-cold methanol/acetonitrile (1:1, v/v). Following incubation at room temperature for 2 h, samples were centrifuged at $12,000 \times g$ for 20 min at 4°C. The supernatant was collected, dried under a gentle nitrogen stream, and reconstituted in 100 μL of methanol/water (3:7, v/v). After vigorous vortexing and a second centrifugation to remove insoluble material, the clarified supernatant was transferred to LC-MS vials for analysis.

Chromatographic separation was performed using both hydrophilic interaction liquid chromatography (HILIC) and reversed-phase columns to enhance metabolite coverage. Mass spectrometric detection was conducted with polarity switching, using positive ion mode (spray voltage, 3.5 kV) and negative ion mode (spray voltage, -3.5 kV). Ion source conditions were set to a heater temperature of 300°C, capillary temperature of 350°C, sheath gas flow of 35 arbitrary units, and auxiliary gas flow of 15 arbitrary units. Metabolite identification was performed using Progenesis QI software (version 2.0; Waters), applying a mass accuracy tolerance of less than 5 ppm against the HMDB serum database.

Pathway analysis and trajectory analysis

Pathway and trajectory analyses were performed to characterize metabolic state transitions from non-cancer controls through pre-invasive LUAD to invasive disease.

Metabolic pathway analysis was conducted using MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca/>).⁷¹ Differential metabolites identified from serum metabolomic profiling were used as input for the integrated *pathway analysis* module, which combines pathway enrichment and topology-based impact assessment.

Pathway enrichment significance was evaluated using a hypergeometric test to determine whether individual pathways were over-represented among the differential metabolites relative to random expectation. The background metabolite set used for enrichment testing is the complete metabolite set of KEGG Homo sapiens database. The `lower.tail` parameter was set to `FALSE` to calculate the probability of observing the given number of significant metabolites or more within each pathway. *p* values were returned in their original scale ($\log_{10} p = \text{FALSE}$) and pathways with $p < 0.05$ were considered significantly altered. To assess the relative importance of metabolites within each pathway, pathway topology analysis was subsequently performed using the relative betweenness centrality metric, which quantifies the extent to which a metabolite functions as a connector within the pathway network.

Metabolic trajectory analysis was performed using fuzzy C-means clustering implemented in the R package `Mfuzz` (version 4.3.1) to identify coordinated patterns of metabolic change across disease stages in the exploratory cohort. Preprocessed mass spectrometry data were used as input, with each sample represented by its consolidated spectral profile. Prior to clustering, data were standardized to zero mean and unit variance to ensure comparability across metabolites. Fuzzy C-means clustering was applied to allow metabolites to exhibit partial membership across clusters, reflecting gradual and overlapping metabolic transitions. The number of

clusters was predefined as four based on empirical evaluation, and the fuzzification parameter controlling cluster softness was determined using the mestimate function. Clustering was iteratively optimized until convergence, yielding stable and reproducible metabolic trajectory patterns.

Machine learning

Diagnostic models were developed using the exploratory cohort and evaluated in independent validation, external validation, and prospective cohorts to assess generalizability. Model development and evaluation were implemented in Python (version 3.7) using the Orange and scikit-learn (sklearn) libraries. A range of supervised learning algorithms was examined, including neural networks, random forests, ridge regression, naïve bayes, support vector machines, adaptive boosting, lasso regression, and gradient boosting. This multi-model framework enabled systematic comparison of algorithmic performance on identical feature sets.

For model training, the NN classifier was selected as the primary model and trained using 10-fold cross-validation within the exploratory cohort to optimize performance and reduce overfitting. All other algorithms were trained under comparable conditions for benchmarking purposes. Hyperparameter optimization for each model was performed using grid search within the cross-validation framework, with optimization objectives defined by maximization of the area under the receiver operating characteristic curve (AUC). Model performance was summarized using cross-validated AUC values.

The optimal diagnostic model was selected based on pairwise comparison of AUCs using the DeLong test, with statistical superiority defined as a significantly higher AUC ($p < 0.05$) relative to all alternative models. Following model selection, the final trained model was applied without retraining to the internal validation, external validation, and prospective cohorts. For classification, a probability threshold of 0.5 was applied to model outputs, a commonly used cutoff that balances sensitivity and specificity. Individuals with predicted probabilities greater than 0.5 were classified as LUAD cases, whereas those with probabilities less than or equal to 0.5 were classified as non-cancer controls.⁷²

Feature selection was performed using a greedy forward-selection strategy implemented in sklearn. The procedure initialized with an empty feature set and iteratively evaluated the incremental contribution of each candidate feature to model performance, quantified by AUC. At each iteration, the feature yielding the greatest improvement in AUC was added to the feature subset. This process was repeated until additional features no longer produced meaningful performance gains, yielding a parsimonious and informative feature set.

During feature selection, model evaluation was conducted using a multilayer perceptron classifier with a single hidden layer of 100 neurons, rectified linear unit (ReLU) activation, and the Adam optimization algorithm. This configuration was selected to provide sufficient model capacity while maintaining computational efficiency and stability during iterative feature evaluation.

Model selection rationale and data leakage control

A NN classifier was selected as the final diagnostic model because it consistently achieved superior discriminative performance during cross-validated training and demonstrated stable generalization across independent validation, external validation, and prospective cohorts. Compared with linear and tree-based models, the NN more effectively captured nonlinear relationships and higher-order interactions among metabolomic features, which are characteristic of high-dimensional serum metabolomic data. Importantly, performance gains were reproducible across cohorts rather than confined to the training set, supporting the robustness of this model choice.

To prevent data leakage, feature selection, model training, and hyperparameter optimization were strictly confined to the exploratory cohort. Feature selection was performed exclusively within cross-validation folds, such that feature ranking and selection were recalculated independently for each training split without access to validation or test data. The resulting optimal feature set was then fixed and applied unchanged to the validation, external validation cohorts. No information from validation samples was used at any stage of feature selection, parameter tuning, or model fitting, ensuring unbiased performance evaluation.

Performance estimation under realistic prevalence assumptions

Since positive predictive value (PPV) and negative predictive value (NPV) are critically dependent on disease prevalence, we performed the estimation under realistic prevalence assumptions to better evaluate the performance of the LUADMet assay in screening. PPV and NPV were calculated as follows⁷³:

$$PPV = \frac{Sensitivity \times Prev}{Sensitivity \times Prev + (1 - Specificity) \times (1 - Prev)}$$

$$NPV = \frac{Specificity \times (1 - Prev)}{Specificity \times (1 - Prev) + (1 - Sensitivity) \times Prev}$$

where, sensitivity and specificity were derived from our assay's performance, which are 84.03% and 80.00%, respectively. Disease prevalence (*Prev*) was derived from the TALENT trial, a large-scale screening study comprising a never-/light-smoking population, which aligns closely with the demographic and risk profile (predominantly female, non-smoking, and East Asian ancestry) in this

study.⁴⁹ Specifically, the cancer prevalence was determined as 14.0% in the LDCT-screened high-risk population and 2.6% in a general screening scenario.

Deployability of LUADMet as a clinical assay

To improve the deployability of LUADMet as a clinical assay, a QC-based procedure was first established to determine how scaling parameters for the 10-metabolite panel are defined and fixed from the training set. In detail, homogeneous pooled QC serum samples were run alongside patient samples for each batch/run. Using these data, we trained a random forest-based QC signal correction (QC-RFSC) model for each of the 10 metabolites.⁷⁴ This model captures the relationship between the fluctuating raw signal of the pooled QC sample (as measured in any run) and its stable, long-term average value. Crucially, the biomarker signals from all training patient samples were then corrected using the predetermined QC-RFSC models, and the final LUADMet classifier was retrained on this corrected dataset. The coefficients of the updated LUADMet model constitute the complete set of fixed parameters for new sample deployment.

Then, to normalize batch effects relative to the training distribution, the same pooled QC sample is analyzed alongside new patient samples. Briefly, the pooled QC sample was introduced for real-time MS instrument monitoring, ensuring that the analytical performance is within the specifications established during model training, thereby guaranteeing that data are acquired under conditions comparable to the LUADMet development. Subsequently, the raw signals for each metabolite, both in a new patient sample and the co-analyzed QC sample, were input into their corresponding pre-trained and fixed QC-RFSC model. The model normalizes the patient sample signal based on the specific deviation observed in the co-analyzed QC sample. Only these QC-RFSC-normalized values are then fed into the fixed LUADMet model for prediction.

QUANTIFICATION AND STATISTICAL ANALYSIS

t-distributed stochastic neighbor embedding (t-SNE) analysis, decision curve analysis, and ROC curve comparisons using the DeLong test were performed in Python (version 3.7) using the scikit-learn and SciPy packages. ROC analyses for CEA and individual metabolic biomarkers were conducted using SPSS software (version 24.0; IBM, USA). Permutational multivariate analysis of variance (PERMANOVA) was performed using R (version 4.3.1; R Foundation).

Normality was assessed using the Shapiro-Wilk test. Non-parametric tests such as the Mann-Whitney U test and Kolmogorov-Smirnov test were applied for two-group comparison, and the Kruskal-Wallis H test for comparisons involving more than two groups. Mann-Whitney U test and Kruskal-Wallis H test were conducted using Python using the SciPy packages. Shapiro-Wilk test and Kolmogorov-Smirnov test were conducted using SPSS. Additional statistical analyses, including the χ^2 test for categorical variables, two-sided Student's *t* test for pairwise comparisons, and one-way analysis of variance (ANOVA) for comparisons across multiple groups, were conducted using SPSS. Unless otherwise specified, all statistical tests were two-sided, and a *p* value < 0.05 was considered statistically significant.