Appendix 1 Metabolic Thermodynamic Sensitivity Analysis (MTSA)

Thermodynamic Analysis and Metabolite Concentration Estimation

The MTSA method begins with determining physiologically relevant metabolite concentrations using the Max/Min Driving Force (MDF) analysis. We assume that each reaction operates at maximum driving force, as all reactions are in pseudo-steady state and occur rapidly. So, for each reaction in our metabolic network, we first parse the reaction formula to identify reactants and products, maintaining stoichiometric coefficients. The MDF analysis employs linear programming optimization to maximize the thermodynamic driving force while satisfying concentration constraints. Metabolite concentrations are constrained between 1 μM and 10 mM, reflecting physiologically relevant ranges. The optimization objective maximizes the minimum driving force across all reactions while ensuring thermodynamic feasibility based on standard Gibbs free energy values (ΔG°) and the calculated RT term for each temperature point. The MDF analysis yields optimal metabolite concentrations that maximize the thermodynamic driving force for each reaction. These concentrations serve as reference points for subsequent kinetic parameter estimation.

Kinetic Parameter Estimation

Following the MDF analysis, we estimate kinetic parameters using the Michaelis-Menten equation. The parameter estimation process involves multiple steps to ensure robust and physiologically relevant results:

Initial Parameter Range Determination

For each reaction, we establish initial parameter ranges based on Flux Variability Analysis (FVA) results. The maximum flux determined by FVA serves as a reference point for Vmax estimation. We consider a range of potential Vmax values spanning from 50% to 150% of this maximum flux, generating five equally spaced estimates within this range. This approach ensures that our parameter search space encompasses physiologically relevant values while accounting for potential variations in enzyme activity.

Synthetic Data Generation

Using the optimal concentrations from MDF analysis, we generate a range of substrate concentrations spanning an order of magnitude below and above the optimal concentration, with 20 logarithmically spaced points. For each substrate concentration, we generate synthetic reaction rate data using the Michaelis-Menten equation, where Vmax is set to the FVA upper bound, and Km is estimated as half the optimal substrate concentration. This estimation of Km as half the optimal concentration is chosen because in the Michaelis-Menten kinetics, Km represents the substrate concentration at which the reaction rate reaches half of its maximum value, making it a reasonable initial approximation for generating synthetic data that reflects typical enzyme kinetic behavior. To simulate experimental variation, we add 30% Gaussian noise to the calculated rates.

Parameter Fitting Process

The Michaelis-Menten equation describes the relationship between reaction rate (v) and substrate concentration ([S]):

$v = (V_{max}[S])/(K_m + [S])$

where:

v is the reaction rate, Vmax is the maximum reaction rate, [S] is the substrate concentration and Km is the Michaelis constant, representing the substrate concentration at which the reaction rate is half of Vmax (see Fig A).

The fitting process employs non-linear regression to fit the Michaelis-Menten equation to the synthetic data.

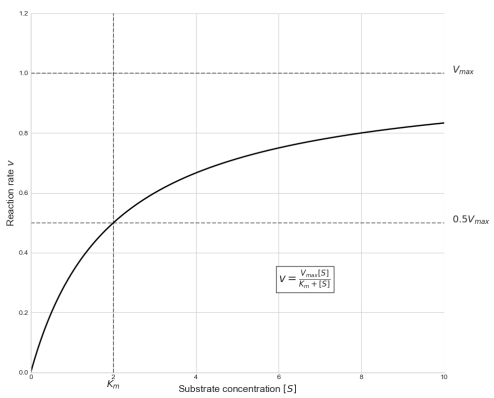

Fig A. Michaelis-Menten equation Profile

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Multiple initial Vmax estimates are tested to avoid local minima in the optimization landscape. For each initial estimate, both Vmax and Km values are determined while enforcing non-negative constraints. The quality of each fit is assessed using the R-squared statistic, and the parameter set yielding the highest R-squared value is selected as the final estimate.

Rate-Limiting Substrate Identification

For reactions involving multiple substrates, we identify the rate-limiting substrate based on the kcat/Km ratio. The substrate exhibiting the lowest kcat/Km ratio is designated as rate-limiting, and its kinetic parameters are used for subsequent calculations. The kcat values are obtained using the DL-Kcat method, which provides enzyme-specific turnover number estimates.

Temperature Dependence Integration

The temperature dependence of metabolic reactions is incorporated through several mechanisms:

1. Temperature effects on reaction thermodynamics are captured through the RT term in the MDF analysis
2. Metabolite concentrations and kinetic parameters are determined separately for each temperature point (36-40 ℃)

Quality Control and Validation

The MTSA method includes several quality control measures to ensure reliable results:

1. Validation of parameter bounds to ensure physically meaningful estimates (positive Vmax and Km values)
2. Assessment of fit quality through R-squared statistics
3. Identification and logging of failed parameter estimations
4. Consistency checks between FVA results and estimated kinetic parameters

This comprehensive approach enables systematic investigation of temperature effects on metabolic reaction rates while maintaining consistency with thermodynamic constraints and network stoichiometry. The method accounts for both the complexity of enzyme-catalyzed reactions and the limitations of available kinetic data, providing robust estimates of temperature-dependent metabolic behavior.

All codes and materials related to MTSA method are available at: https://github.com/ssbio/MTSA.git

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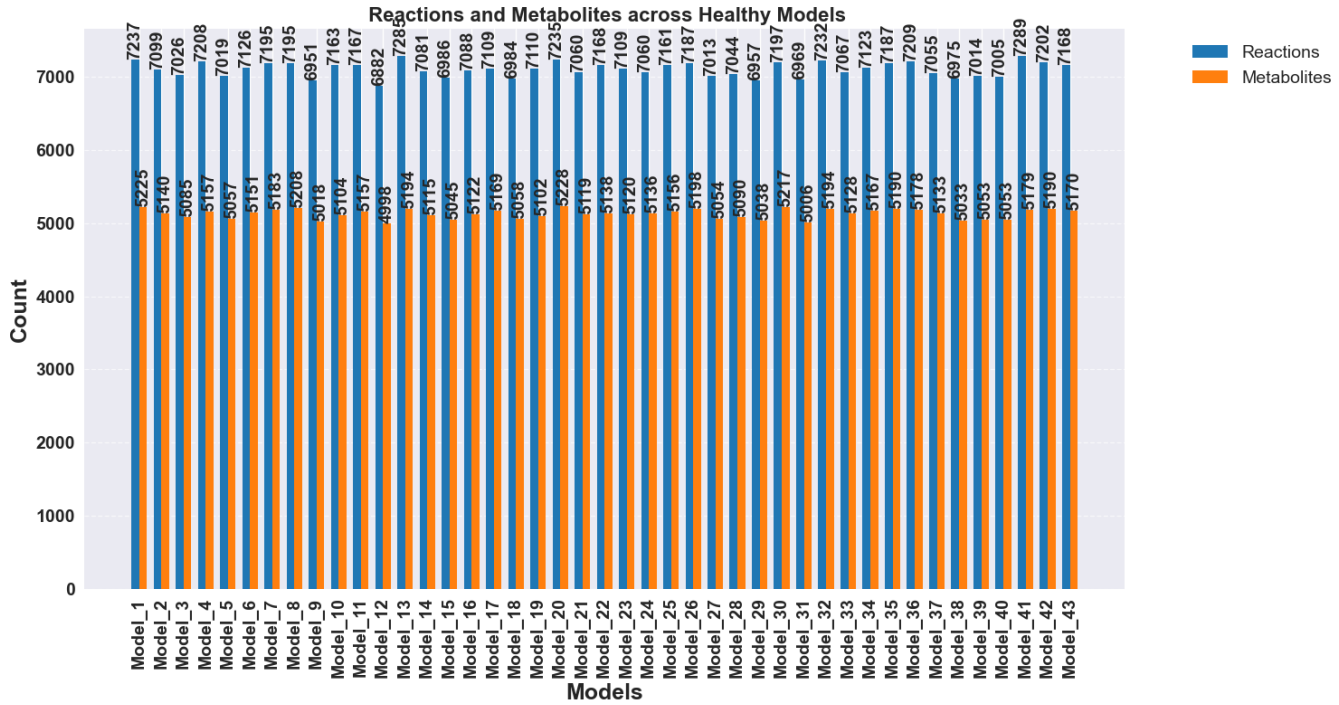

Figure S1 The distribution of reactions and metabolites across healthy lung tissue models.

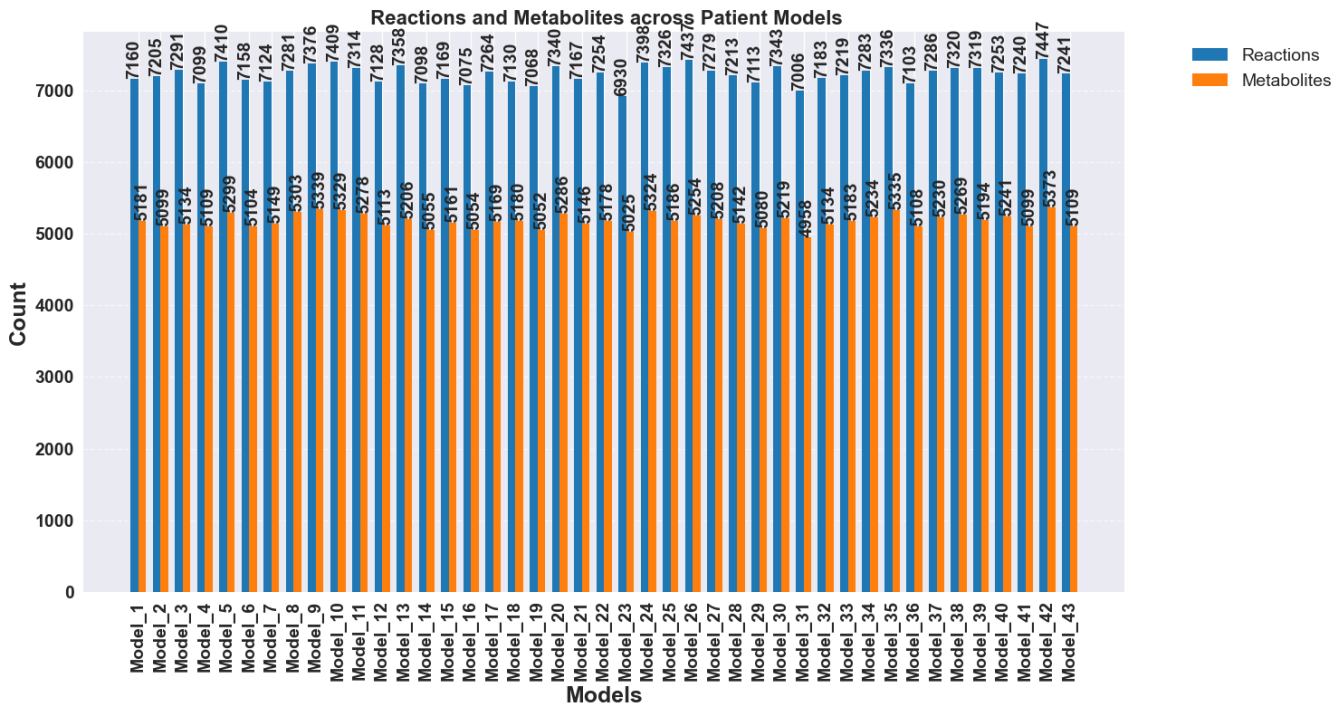

Figure S2 The distribution of reactions and metabolites across cancerous lung tissue models.

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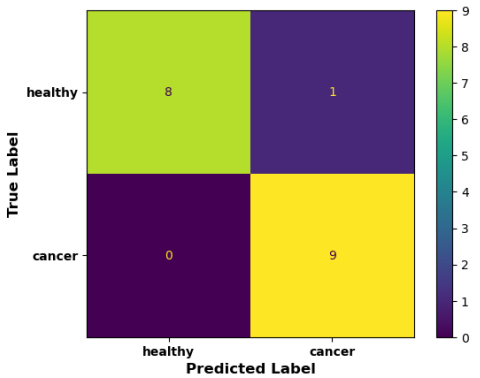
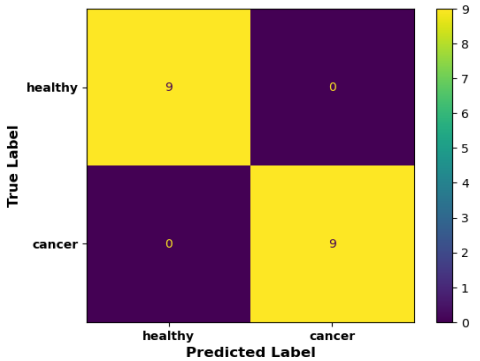
Table S1 Performance comparison of Random forest classifiers for Lung Bulk Tissue and mast cell metabolic models

Model	Mean Accuracy	Precision	Recall	F1-Score	Confusion Matrix
Lung Bulk Tissue Models	0.95	0.95	0.94	0.94	
Mast Cells Models	1.0	1.0	1.0	1.0	

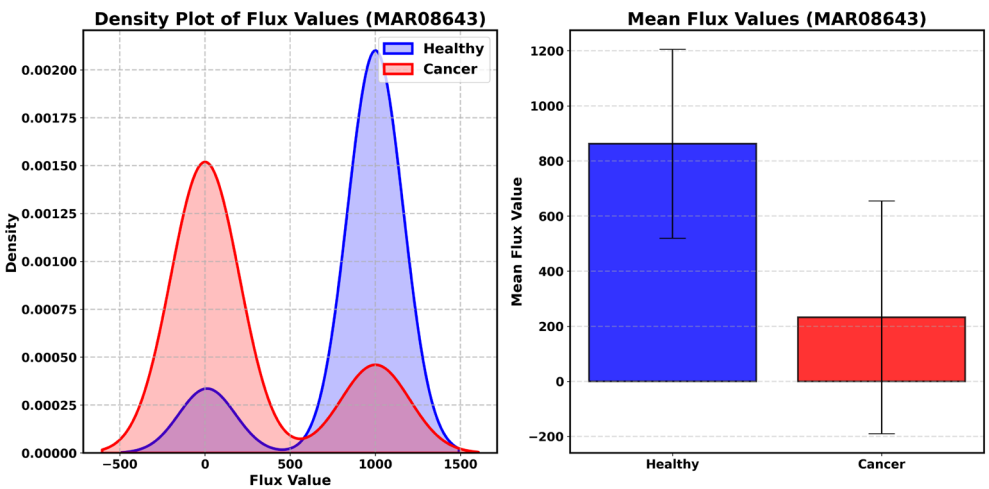


Figure S3 Analysis of metabolic flux distributions in MAR08643 reaction.

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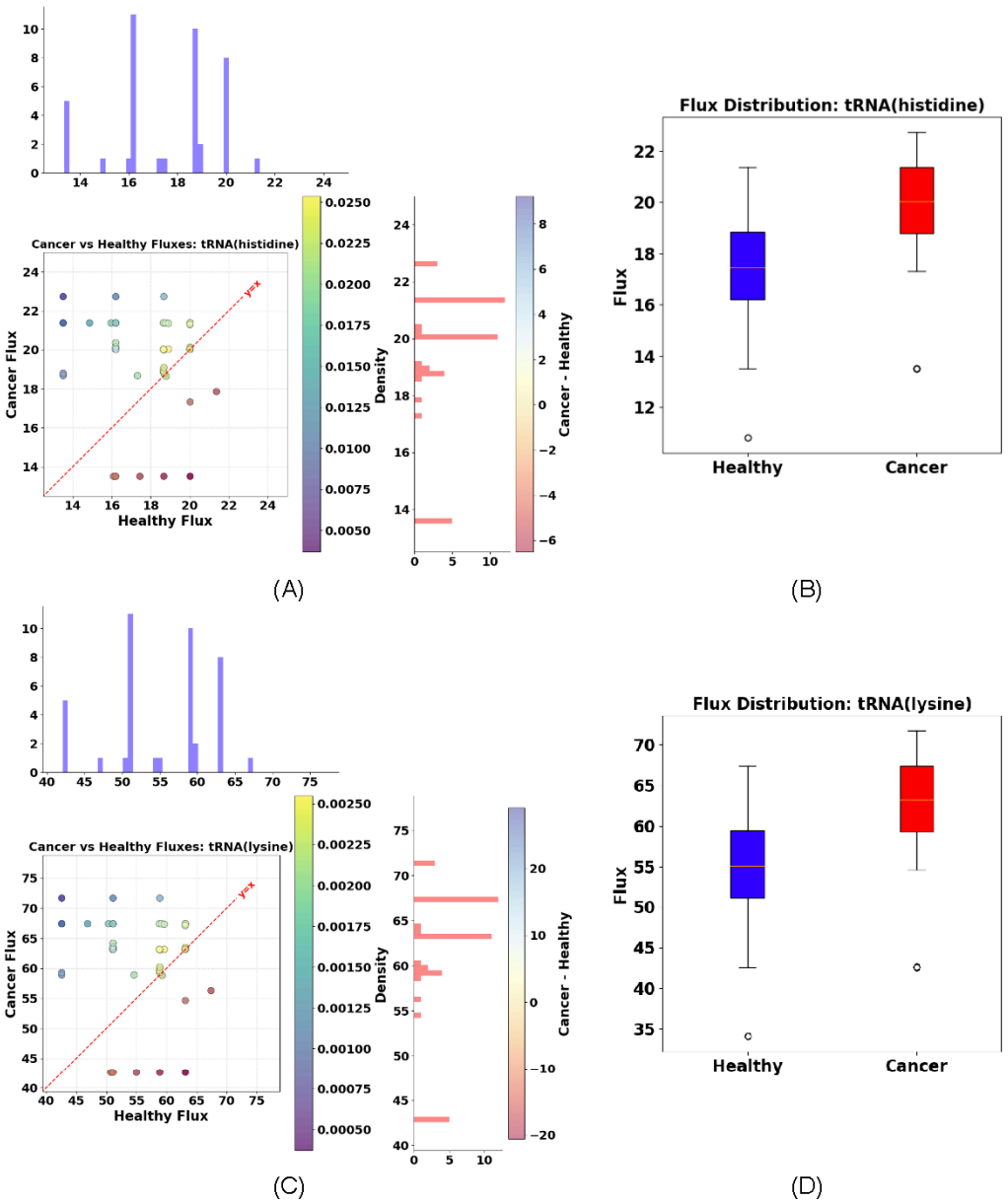

Figure S4 Analysis of metabolic flux distributions in aminoacyl-tRNA synthesis pathway between cancer and healthy lung tissues. (A) Flux comparison between healthy and cancer samples for the histidine aminoacylation reaction, including scatter plot (left) with y=x reference line and density distribution (right). (B) Box plot showing the flux distribution comparison between healthy and cancer samples for tRNA(his) synthesis. (C) Flux comparison between healthy and cancer samples for the lysine aminoacylation reaction, including scatter plot (left) with y=x reference line and density distribution (right). (D) Box plot showing the flux distribution comparison between healthy and cancer samples for tRNA(lys) synthesis. Both reactions demonstrate differences in flux values between cancer and healthy tissue samples, potentially indicating altered aminoacyl-tRNA synthesis activity in lung cancer metabolism.

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