SUPPLEMENTAL MATERIALS

A novel 6-metabolite signature for prediction of clinical outcomes in type 2 diabetic patients undergoing percutaneous coronary intervention

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Supplementary Methods

1. Baseline and outcome data

1.1 Diagnosis of type 2 diabetes mellitus (T2DM)

T2DM was diagnosed based on the 2014 criteria of the American Diabetes Association: fasting plasma glucose ≥ 7.0 mmol/L or 2-h postchallenge plasma glucose ≥ 11.1 mmol/L or glycated hemoglobin ≥ 6.5% or ongoing therapy for T2DM [1].

1.2 Definition of clinical characteristics

Current smokers were defined as having smoked ≥ 100 cigarettes in their lifetime and now smoking every day or some days. Hypertension was defined as ongoing therapy for hypertension, systolic blood pressure of ≥ 140mmHg or diastolic blood pressure of ≥ 90mmHg. Dyslipidemia was defined as hypercholesterolemia (serum total cholesterol > 5.72 mmol/L), high levels of low-density lipoprotein cholesterol (> 3.1 mmol/L), low levels of high-density lipoprotein cholesterol (< 0.9 mmol/L), and hypertriglyceridemia (serum triglyceride > 1.70 mmol/L). Peripheral vascular disease was defined as arteries other than coronaries, with exercise-related claudication, revascularization surgery, reduced or absent pulsation, and/or angiographic stenosis of > 50%. Left ventricular end-systolic and end-diastolic volumes were measured using a standard ultrasound machine with a 2.5-MHz probe, and left ventricular ejection fraction was calculated by Simpson biplane method [2].

1.3 Angiographic characteristics

Before primary percutaneous coronary intervention (PCI), all participants
underwent invasive coronary angiography using the Judkins percutaneous trans-femoral technique. Digital angiograms were reviewed by two expert observers to document lesion characteristics. From the angiograms, moderate (readily visible but mild degree) or severe (obvious, heavy degree) calcification was recorded [3]. Chronic total occlusion was defined as a luminal occlusion in a native coronary artery with no or minimal contrast penetration through the lesion. Multivessel coronary artery disease (CAD) was defined as coronary artery stenosis of $\geq 50\%$ in at least 2 major epicardial coronary arteries. The anatomical complexity of CAD was assessed using the SYNTAX score [4]. Interobserver variability between 2 observers was 0.84 (0.77–0.91), and intraobserver variability was 0.96 (0.93–0.98).

1.4 Study outcome

The primary outcome for all datasets was major adverse cardiovascular events (MACEs), a patient-oriented composite endpoint composed of all-cause death, myocardial infarction (MI), stroke, and repeat revascularization. All-cause death was defined as death from any cause. MI was defined as ischaemic signs or symptoms and new pathological Q-waves in $\geq 2$ contiguous ECG leads, or/and an elevation CK-MB or troponin above the 99th percentile limit of normal and at least $\geq 20\%$ above the most recent value. Stroke was defined as a focal neurologic deficit of central origin lasting $> 72$ h, or a focal neurologic deficit of central origin lasting $> 24$ h, with imaging evidence of cerebral infarction or intracerebral hemorrhage. Repeat revascularization was defined as any repeat PCI. All stages of a staged index PCI procedure would be considered part of the index revascularization procedure and not a
repeated revascularization.

The secondary outcome of the external validation set was target lesion failure, a device-oriented composite endpoint of cardiac death, target vessel-MI, and target lesion revascularization. Cardiac death was defined as any death due to proximate cardiac cause (MI, significant cardiac arrhythmia, refractory congestive heart failure, etc), procedure-related death, and death of unclear cause [5]. Target lesion revascularization was defined as any repeat PCI of the target lesion as a result of restenosis or other complications of the target lesion.

2. Untargeted metabolomic profiling

2.1 Serum sample preparation

At first, 180 µL of serum samples was mixed with 800 µL of acetonitrile/methanol (1:1, v/v) and 20 µL of internal standard (L-2-Chlorophenylalanine, 2 µg/mL). Then, the mixture was vortexed for 30 s, sonicated for 10 min, and incubated for 1 h at -20 °C to precipitate proteins. After centrifugation at 13,000 g for 15 min at 4 °C, the supernatant (800 µL) of the mixture was carefully collected, dried under nitrogen, and resuspended in 200 µL of 80% methanol before analysis.

2.2 LC-MS analysis

Serum metabolomic profiling was performed on a Vanquish UHPLC system (Thermo, Waltham, USA) coupled with a Q Exactive Orbitrap mass spectrometer (Thermo, Waltham, USA) operating in positive (ESI+) and negative (ESI-) modes. Samples were first eluted on an UPLC BEH Amide column (2.1 mm × 100 mm, 1.7
μm) at 25 °C with a flow rate of 0.3 mL/min. The mobile phase A was ultrapure water (pH: 9.75) containing 25 mM NH₄OH and 25 mM NH₄OAc and the mobile phase B was 25 mM acetonitrile. The gradient program was: 1) 0-0.5 min, 95% B; 2) 0.5-7.0 min, 95%-65% B; 3) 7.0-8.0 min, 65%-40% B, 4) 8.0-9.0 min, 40% B; 5) 9.0-9.1 min, 40%-95% B; 6) 9.1-12.0 min, 95% B. The auto-sampler temperature was 4 °C, and the injection volume was 3 μL.

To acquire MS/MS spectra, the column eluent was further detected by means of information-dependent acquisition on a Q Exactive Orbitrap Mass Spectrometer. The scan range was 60-1200 Da. The ion source parameters were as follows: sheath gas flow rate, 30 Arb; auxiliary gas flow rate, 25 Arb; capillary temperature, 350 °C; full MS resolution, 60000; collision energy, 10, 30, or 60 V in ESI+ and -10, -30, or -60 V in ESI- modes, spray Voltage, 3.6 kV (ESI+) and -3.2 kV (ESI-).

3. Targeted metabolite analysis

3.1 Serum sample preparation

Briefly, 100 μL of serum samples was extracted in 400 μL of ice-cold methanol, centrifuged for 13,000 g at 4 °C for 10 min, and filtered through 3 kDa membrane cartridges. Sample extracts were then dried under vacuum, reconstituted in 200 μL of 100 mM NH₄OAc buffer, and capped before analysis.

3.2 LC-MS analysis

The targeted metabolite analysis of the 6 selected metabolites was conducted by a 20AD UPLC system (Shimadzu, Kyoto, Japan) coupled with a QTrap 5500 mass spectrometer (SCIEX, Framingham, USA), as described previously.
Samples and standards (20 μL) were injected onto a Phenom-exex NH₂ column (150 mm × 2 mm × 3 μm) for metabolite separation. A binary solvent gradient consisting of 5 mM NH₄OAc (pH: 9.5) adjusted with ammonia (mobile phase A) and acetonitrile (mobile phase B) with a flow rate of 0.25 mL/min. Initial solvent composition at injection was 25% A, followed by a 2-min gradient to 45% A and a fast gradient ramp to 80% A (0.1 min) which was maintained for 5.9 min, A was increased again to 95% (2 min), held for 13 min and then reverted to initial conditions (0.1 min) for equilibration, with a total run time of 30 min.

The column flow was directed into the MS detector operated in the multiple reaction monitoring mode. The ion source parameters were as follows: sheath gas flow rate, 20 Arb; auxiliary gas flow rate, 10 Arb; capillary temperature, 300 °C; capillary voltage, 4000 V. Other MS/MS settings, such as declustering potential and collision energy, were optimized for each particular metabolite. The concentrations of detected metabolites were obtained from 7-point calibration curves, which were constructed using the peak area ratios (peak area of the metabolite divided by peak area of the isotope-labeled internal standards) of each calibrator versus its concentration. All calibrators were purchased from Sigma (cat # 72340, cat # M4627, cat # C7344, cat # C0254, cat # P2263). All the involved internal standards, including NAM-D4 (for NAM, 1-MNAM, and ADPR, cat # DLM-6883-PK), L-tryptophan-D5 (cat # DLM-1092-PK), D-glucose-6-phosphate-C13 (for D-ribose-5-phosphate, cat # CLM-9601-PK), and 1,2-diheptanoyl-sn-glycero-3-phosphocholine-D26 (for PC(36:2), cat # DLM-11092-PK), were purchased from Cambridge Isotope
Laboratories. The calibration data of each detected metabolite, including calibration range, calibration linearity, regression equation, precision, and accuracy, were summarized in Table S1.

4. Machine learning algorithms

4.1 Random forest (RF)

The R package "randomForest" was used for this algorithm. A RF model of the 6 metabolic predictors was trained with 10-fold cross-validation. The 2 important RF parameters, nTree (number of trees to grow for each forest) and mTry (number of predictors sampled for splitting at each node), were set to 500 and the default setting, respectively.

4.2 Extreme Gradient Boosting (XGBoost) tree

The R package "xgboost" was used for this algorithm. An XGBoost tree model of the 6 metabolic predictors was trained with 10-fold cross-validation. Key parameters were set as follows: eta, 0.05; max_depth, 20; min_child_weight, 1 (default); nrounds, 100; objective, "binary: logistic"; eval_metric, "auc".

4.3 Support Vector Machines (SVM)

The SVM model was fitted with the R package "e1071". The kernel parameter was set as "polynomial", which showed the best predictive performance on the discovery set among all kernel types. We set the "cost" parameter as 0.3, the "gamma" parameter as 1, the "degree" parameter as 4, and the "coef0" parameter as 1.

4.4 Deep neural network (DNN)
We trained a deep, multilayer neural network with 2 hidden layers using the R package "keras". We used a rectified linear unit function as the non-linear activation function and a sigmoid function as the classification function. Model optimization was guided using the adaptive moment estimation optimizer. The hyperparameters were manually tuned as follows: regularization factor, 0.002; learning decay rate for the first momentum, 0.9; for the second momentum, 0.999; batch size, 1024; epochs, 30. The DNN was designed as having 2 hidden layers with 32 input units in the first layer and 16 input units in the second layer. Ten percent of the input units were used as the drop-out layers, which evaluate the cross-entropy loss function over the number of epochs and reduce the learning rate when the loss function stopped improving.

5. **In vitro experiments**

5.1 **NAD⁺ detection by LC-MS**

Prior to NAD⁺ detection, high glucose-cultured HASMCs (LONZA, cat # CC-2571) were trypsinised, washed, and seeded to 24-well plates at 10⁵ cells per well. The processes of sample preparation, LC separation, and MS detection were the same as the target metabolite analysis mentioned above. The isotope-labeled internal standard used for NAD⁺ detection was adenosine monophosphate C13 (Cambridge Isotope Laboratories, cat # CNLM-3802-SL-10), as described by Grant et al [6].

5.2 **Determination of mitochondrial complex activity**

HASMCs were fractionated by mitochondria isolation kit (Sigma, cat # MITOISO2-1KT). The activity of mitochondrial respiratory chain complexes (I-V) in mitochondria fractions was determined by spectrophotometric methods summarized
5.3 Bioenergetic profiles detected by the Seahorse technology

The Seahorse XFe96 Analyzer (Agilent, Santa Clara, USA) was used to simultaneously detect mitochondrial respiration and glycolysis of HAoSMCs by generating the time course of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), respectively. At first, HASMCs (6 × 10^5 cells per well) were plated into a Seahorse XF24 Cell Culture Microplate (Agilent) filled with high glucose DMEM, and treated with 10 μM FK866 (Sigma, cat # F8557), 200 μM 1MT (Sigma, cat # 447439), or 10 μM NMN (Sigma, cat # N3501) for 20 h.

For measurement of mitochondrial respiration, the medium was replaced with DMEM containing 1 mM pyruvate, 2 mM glutamine, and 25 mM glucose prior to assays. Then, cells were sequentially exposed to 1 μM oligomycin (Oligo, Abcam Biochemicals, cat # 1404-19-9), 0.75 μM FCCP (Sigma, cat # C2920), and a mix of 1 μM each rotenone (Sigma, cat # R8875) and antimycin (Sigma, cat # A8674) (R + A) at the indicated times, and the OCRs of HASMCs were measured over time (as presented in Fig. 5C). According to the manufacturer’s protocol, non-mitochondrial OCR was calculated as OCR after R + A injection; Basal respiration was calculated as the difference between the OCR before Oligo injection and non-mitochondrial OCR; ATP-linked respiration was calculated as the difference between basal and Oligo-inhibited OCR. Maximal respiration was calculated as the difference between FCCP-induced OCR and non-mitochondrial respiration. Reserve respiratory capacity was calculated as the difference between maximal and basal respiration.
For measurement of glycolytic flux, the medium was replaced with glucose-free DMEM prior to assays. Then, cells were sequentially exposed to 19 mM glucose, 1 µM Oligo, and 50 mM 2-deoxy-glucose (Sigma, cat # D6134) at the indicated times (as presented in Fig. 5E), and the ECARs of cells were measured over time. According to the manufacturer’s protocol, non-glycolytic acidification was calculated as ECAR before glucose injection; glycolysis was calculated as the difference between glucose-induced ECAR and non-glycolytic acidification; glycolytic capacity was calculated as the difference between Oligo-induced ECAR and non-glycolytic acidification; glycolytic reserve was calculated as the difference between glycolytic capacity and glycolysis. All the readings of OCRs and ECARs were normalized to the total protein content of each well (determined by the Bradford method [8]).

5.4 Reverse-transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from HASMCs using the Trizol reagent (Sigma, cat # 93289). cDNA was synthesized with RNA to cDNA EcoDry Premix (Clontech, cat # 639547). The mRNA expression of proinflammatory genes was determined by RT-qPCR on a CFX96 Touch system (Bio-rad, Hercules, USA). RT-qPCR was performed in triplicate based on the MIQE guidelines [9]. The relative expression of a target gene was normalized to the expression of reference gene (GAPDH) using the \(2^{-\Delta\Delta C_q}\) method [10]. The calibrator was a cDNA sample from the control group. The primers used for RT-qPCR analysis are listed in Table S2.

5.5 Transwell migration assay

HASMCs were seeded into 24-well plates, with administration of FK866, 1MT,
or NMN as mentioned above. Transwell inserts with 8 μm pores (Sigma, cat # CLS9668) were pre-coated with 10 μg/mL fibronectin for 24 h, and applied in suspension. THP1 monocytes were labeled with Qdot (Invitrogen, cat # Q10141MP) following the manufacturer's instructions, and then added to the top of each transwell. After 4 h incubation, transwell inserts were taken, and un-migrated monocytes on the upper membrane were removed by gentle rubbing with a cotton bud. Migrated Qdot-labeled THP1 cells were enumerated by fluorescence microscopy.

5.6 Proliferation assay

HASMC proliferation was assessed by a methylene blue assay [11]. Briefly, HASMCs (1.5 × 10^5 cells/well) were placed in sterile 96-well culture plates and cultured in DMEM, with administration of FK866 (10 μM), 1MT (200 μM), or NMN (10 μM) for 20 h. Cells were then fixed in 10% formaldehyde saline for 30 min, and incubated with 1 % (w/v) methylene blue in 0.01 mM borate buffer (pH 8.5) for 30 min. The remaining dye was washed off by serially dipping the plate into each of four tanks of 0.01 mol/L borate buffer (pH 8.5). The resulting cell growth was measured at 540 nm and calculated in comparison with the growth of the control group.
Supplementary Figures

**Figure S1** The Z distribution of methionine abundance in both discovery and internal validation sets. The Z scores of methionine across all samples were greater than -3 (-3SD), indicating that the serum samples were properly stored for metabolite detection.

**Figure S2** The Pearson correlation between metabolic data of quality control samples assessing the reliability of the LC-MS analysis in the discovery (A) and internal validation sets (B).
**Figure S3** The OPLS-DA analysis assessing the performance of 35 differential metabolites for discrimination of MACEs from matched controls in the discovery (A) and internal validation sets (B).

**Figure S4** The differences in cumulative rates of MACEs between participants with high-risk and low risk scores of the 6-metabolite signatures. *P* values were derived from Cox regression with adjustment for age, sex, smoking status, obesity (BMI > 25 kg/m²), hypertension, HbA1c, LVEF < 50%, clinical presentations, multivessel CAD, SYNTAX score, and stent types.
**Figure S5** Scatter plots for comparing the predictive performance of the random forest (A) and XGBoost (B) models to the logistic regression model of the 6-metabolite panel. The models are generated using the discovery dataset and presented here in the internal validation set. Red lines indicate the cut-offs of random forest and XGBoost models; Black lines indicate the cut-off of logistic regression model. Black circles label MACEs that would be identified using the random forest and XGBoost models but would be missed when the logistic regression model is applied.

**Figure S6** The importance of each predictor of the 6-metabolite classifier constructed by random forest (A) and XGBoost (B). Abbreviations: NAM, nicotinamide; ADPR, adenosine diphosphate ribose; 1-MNAM, 1-methylnicotinamide; PC, phosphatidylcholine.
**Figure S7** Calibration plots for the logistic regression and 4 machine learning models of the 6-metabolite classifier in the discovery (A) and internal validation sets (B). Abbreviations: RF, random forest; XGBoost, extreme gradient boosting; SVM, Support Vector Machines; DNN, deep neural network.

**Figure S8** Backward stepwise Cox regression analyses of the association between the 6-metabolite classifier and MACEs in the external validation set. (A) The log-minus-log plot for graphically testing the proportional hazards assumption. (B) Kaplan-Meier curve for assessing the performance of the 6-metabolite classifier to predict MACEs. In the backward stepwise Cox regression analyses, variables including age, sex, smoking status, obesity (BMI > 25 kg/m²), hypertension, HbA1c, LVEF < 50%, clinical presentations, multivessel CAD, SYNTAX score, stent types, and the 6-metabolite classifier were first entered one at a time. Then, 4 variables with $P < 0.10$ (i.e. HbA1c, LVEF < 50%, multivessel CAD, and the 6-metabolite classifier) in the stepwise procedure were retained to fit the final model. The HR and $P$ value were calculated accordingly.
**Table S1**

Calibration data for 6 metabolites detected by targeted metabolite analyses.

| Metabolites          | Calibration range (µmol/L) | Regression equation       | Linearity (r²) | Intra-batch precision (%) | Inter-batch precision (%) | Accuracy (%) |
|----------------------|-----------------------------|---------------------------|----------------|---------------------------|---------------------------|--------------|
| ADPR                 | 0-0.8                       | \( y = 5.0 \times 10^{-3}x + 7.6 \times 10^{-1} \) | 0.9979         | 9.6                       | 5.4                       | 4.1          |
| 1-MNAM               | 0-0.8                       | \( y = 8.4 \times 10^{-3}x + 7.0 \times 10^{-1} \) | 0.9938         | 13.7                      | 8.8                       | -4.2         |
| D-Ribose 5-phosphate | 0-2.1                       | \( y = 4.7 \times 10^{-3}x + 1.6 \times 10^{-2} \) | 0.9958         | 5.8                       | 3.4                       | -9.1         |
| NAM                  | 0-0.8                       | \( y = 7.5 \times 10^{-3}x + 8.2 \times 10^{-3} \) | 0.9937         | 6.0                       | 3.8                       | 3.4          |
| L-Tryptophan         | 10-510                      | \( y = 8.3 \times 10^{-5}x + 5.4 \times 10^{-5} \) | 0.9942         | 13.1                      | 7.6                       | -2.0         |
| PC(36:2)             | 0-640                       | \( y = 6.8 \times 10^{-3}x + 1.8 \times 10^{-1} \) | 0.9961         | 10.4                      | 6.4                       | 5.3          |

Abbreviation: ADPR, adenosine diphosphate ribose; 1-MNAM, 1-methylnicotinamide; NAM, nicotinamide; PC, phosphatidylcholine
### Table S2

List of primers used in RT-qPCR.

| Gene  | Primers Forward | Primers Reverse | Length (bp) | Ann. Temp (°C) |
|-------|-----------------|-----------------|-------------|----------------|
| MCP1  | GCTCAGCCAGATGCAATCA | TGGAAATCTGAAACCCTTT | 193 | 56 |
| CCL3  | CCCTTGCTGTCCTCCTCTG | GGTCGCTGACATATTTCCTG | 243 | 58 |
| CCL4  | AGCCCGGATGCTTCTCCA | CTAATACAAATAACACGGCACATAA | 113 | 58 |
| IL6   | GCCCTGCGTCCGATTTCC | CTTTTCTGTTCCCCTGGG | 179 | 60 |
| IL8   | TTGGCAAGCCTCCCTAGATT | CCTTGGGTCACGACAGAAG | 217 | 58 |
| IL1β  | GATGGCTTATTACAGTGCA | GTAGTGTTGGTGCGAGATT | 139 | 56 |
| CCL7  | TTCAACTACTCTGCTTCA | ATAAAAGTCTTGGACCCACT | 171 | 54 |
| CXCL2 | AACCGAAGTCATAGCCACA | AGAACAGCACCACAATAAAG | 149 | 56 |
| CX3CL1| TGTAGCTTTTGCTCACATCCACTATC | AACCAGACACTATCCACTATCC | 246 | 58 |
| IL1α  | TGTATGTGACTGCCCAAGA | TGAGACCTGAGGCTACAGCC | 258 | 56 |
| TNFα  | CCCATGTGTAGCAAAACCC | TGGTAGGAGACGGCGATGC | 223 | 58 |
| PTGS2 | CTTCCCTCTTGCCCTAGTG | ACTGATGGTGGAATGGCTT | 172 | 58 |
| GAPDH | GAAGGTGAAAGGTCGGAGTC | GAAGATGGTGGAATGGTAC | 226 | 58 |

(Reference)

### Table S3

Ontology classes of metabolites in the discovery and internal validation sets.

| Ontology class                      | Discovery set (n = 743) | Internal validation set (n = 674) |
|-------------------------------------|-------------------------|-----------------------------------|
| Lipids, fatty acids and related     | 320                     | 305                               |
| Energy metabolism and related       | 100                     | 93                                |
| Carbohydrates and related           | 94                      | 86                                |
| Amino acids and related             | 88                      | 74                                |
| Peptides, and analogues             | 37                      | 29                                |
| Signal substances and related       | 34                      | 30                                |
| Vitamins and related                | 27                      | 21                                |
| Nucleosides and related             | 26                      | 20                                |
| Alkaloids and derivatives           | 9                       | 9                                 |
| Miscellaneous                       | 8                       | 7                                 |
| ID | Metabolite name                                      | Subpathway                        | Superpathway                  | VIP | P-VALUE      | FDR     | Fold Change |
|----|-----------------------------------------------------|-----------------------------------|-------------------------------|-----|--------------|---------|-------------|
| 1  | Indolepyruvate                                      | Tryptophan metabolism             | Amino acid                    | 2.22 | 1.09E-03    | 9.99E-03 | 5.36        |
| 2  | Acetate                                             | Glycolysis / Gluconeogenesis      | Carbohydrates and related     | 2.04 | 2.07E-03    | 9.77E-03 | 4.62        |
| 3  | ADPR                                                | Nicotinate and nicotinamide       | Energy metabolism             | 2.64 | 5.49E-10    | 1.06E-07 | 4.61        |
| 4  | beta-D-Glucoside                                    | Starch and sucrose metabolism     | Carbohydrates and related     | 2.51 | 7.13E-08    | 5.04E-06 | 3.88        |
| 5  | Choline sulfate                                     | ABC transporters                  | Signal substances and related | 1.68 | 3.20E-03    | 1.35E-02 | 3.63        |
| 6  | Tryptamine                                          | Tryptophan metabolism             | Amino acid                    | 1.24 | 1.11E-03    | 6.07E-03 | 2.59        |
| 7  | L-Serine-phosphoethanolamine                        | Glycerophospholipid               | Lipids                        | 2.20 | 7.49E-07    | 2.13E-05 | 2.29        |
| 8  | (S)-Lactate                                         | Glycolysis                        | Carbohydrates and related     | 1.62 | 2.86E-06    | 5.93E-05 | 2.22        |
| 9  | 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid   | Fatty acid metabolism             | Lipids                        | 1.51 | 9.47E-03    | 2.98E-02 | 2.19        |
| 10 | 2,6-Dihydroxy pyridine                             | Nicotinate degradation            | Energy metabolism             | 2.16 | 1.68E-04    | 1.45E-03 | 1.99        |
| 11 | Desglucochoerotoxin                                | Steroid lactones                  | Lipids                        | 2.09 | 4.32E-04    | 2.96E-03 | 1.94        |
| 12 | 5-Amino pentanamide                                | Lysine degradation                | Amino acid                    | 2.12 | 5.58E-04    | 3.57E-03 | 1.86        |
| 13 | LysoPC(C18:1)                                       | Glycerophosphocholines            | Lipids                        | 1.41 | 5.57E-05    | 6.08E-04 | 1.86        |
| 14 | 5-Carboxamide                                       | Nicotinate and nicotinamide       | Energy metabolism             | 2.22 | 1.55E-03    | 7.83E-03 | 1.85        |
| 15 | 1-MNAM                                              | Nicotinate and nicotinamide       | Energy metabolism             | 2.56 | 5.53E-09    | 7.79E-07 | 1.80        |
| 16 | Phenylacetylglutamine                               | Phenylalanine metabolism          | Amino acid                    | 1.06 | 9.15E-03    | 2.90E-02 | 1.79        |
| 17 | Indole-3-acetamide                                 | Tryptophan metabolism             | Amino acid                    | 2.02 | 1.75E-06    | 4.04E-05 | 1.71        |
| 18 | Oleic acid                                          | Fatty acid metabolism             | Lipids                        | 1.98 | 6.16E-04    | 3.84E-03 | 1.63        |
| 19 | dUMP                                                | Pyrimidine deoxyribonucleotides   | Nucleosides and related        | 1.84 | 9.83E-05    | 9.49E-04 | 1.62        |
| 20 | PA(34:1)                                            | Phosphatidic acid                 | Lipids                        | 2.09 | 1.10E-05    | 1.65E-04 | 1.59        |
| 21 | Indole-3-acetate                                    | Tryptophan metabolism             | Amino acid                    | 1.83 | 8.23E-05    | 8.29E-04 | 1.57        |
| 22 | D-Ribose 5-phosphate                               | Pentose phosphate                 | Carbohydrates and related     | 2.73 | 1.27E-10    | 3.25E-08 | 1.57        |
| 23 | Pyruvic acid                                        | Glycolysis / Gluconeogenesis      | Carbohydrates and related     | 1.64 | 7.44E-05    | 7.66E-04 | 1.57        |
| 24 | Arachidonic acid                                   | Fatty acid metabolism             | Lipids                        | 1.12 | 1.46E-02    | 4.06E-02 | 1.57        |
| 25 | PC(33:3)                                            | Phosphatidylinositol              | Lipids                        | 1.50 | 1.09E-03    | 5.97E-03 | 1.54        |
| 26 | Cysteine-S-sulfate                                 | Cysteine and methionine metabolism| Amino acid                    | 1.88 | 2.25E-04    | 1.81E-03 | 1.50        |
| 27 | 2-Methylguanosine                                  | Purine nucleosides                | Nucleosides and related        | 1.82 | 3.76E-03    | 1.52E-02 | 1.45        |
| 28 | D-ribose                                           | Pentose phosphate                 | Carbohydrates and related     | 1.03 | 9.89E-03    | 3.07E-02 | 1.44        |
| 29 | PC(34:2)                                            | Phosphatidylinositol              | Lipids                        | 2.02 | 2.25E-05    | 2.96E-04 | 1.43        |
| 30 | 3-Phospho-D-glycerate                              | Pentose phosphate                 | Carbohydrates and related     | 1.88 | 4.00E-03    | 1.59E-02 | 1.42        |
| 31 | Lactosylceramide (d18:1/16:0)                       | Glycosphingolipids                | Lipids                        | 1.68 | 1.32E-03    | 6.94E-03 | 1.40        |
| No. | Compound                                      | Pathway                                           | Type                                      | Z-score | Log2FoldChange | p-value         | q-value         | Adj.p-value       |
|-----|-----------------------------------------------|---------------------------------------------------|-------------------------------------------|---------|---------------|----------------|----------------|--------------------|
| 32  | Propionic acid                                | Nicotinate and nicotinamide metabolism            | Energy metabolism                        | 1.93    | 7.58E-05      | 7.77E-04       | 1.40           |                    |
| 33  | 8-Methoxykynurenate                           | Tryptophan metabolism                             | Amino acid                                | 1.05    | 1.38E-02      | 3.89E-02       | 1.40           |                    |
| 34  | PC(33:4)                                      | Phosphatidylcholine                               | Lipids                                    | 2.47    | 1.11E-06      | 2.77E-05       | 1.38           |                    |
| 35  | Dihydrolipoate                                | Fatty acid metabolism                             | Lipids                                    | 2.14    | 1.17E-03      | 6.31E-03       | 1.35           |                    |
| 36  | L-Acetylcarnitine                             | Fatty acid metabolism                             | Lipids                                    | 1.36    | 4.19E-03      | 1.64E-02       | 1.34           |                    |
| 37  | Hexadecylglycerol-3-phosphocholine            | Glycerophosphocholines                            | Lipids                                    | 2.40    | 3.98E-07      | 1.42E-05       | 1.34           |                    |
| 38  | Nicotinamide riboside                         | Nicotinate and nicotinamide metabolism            | Energy metabolism                        | 2.18    | 1.85E-05      | 2.51E-04       | 0.74           |                    |
| 39  | 7-Dehydrodesmosterol                          | Cholesterolan steroids                            | Lipids                                    | 1.50    | 1.13E-05      | 1.68E-04       | 0.74           |                    |
| 40  | PC(38:3)                                      | Phosphatidylcholine                               | Lipids                                    | 1.94    | 1.23E-04      | 1.13E-03       | 0.74           |                    |
| 41  | 3-Methylhistidine                             | Histidine metabolism                              | Amino acid                                | 1.86    | 1.59E-04      | 1.39E-03       | 0.73           |                    |
| 42  | Nicotinate                                    | Nicotinate and nicotinamide metabolism            | Energy metabolism                        | 1.93    | 7.64E-04      | 4.54E-03       | 0.73           |                    |
| 43  | PE(40:4)                                      | Phosphatidylethanolamine                          | Lipids                                    | 1.86    | 5.05E-04      | 3.31E-03       | 0.73           |                    |
| 44  | SM(d18:0/14:0)                                | Phosphatidylglycerol                              | Lipids                                    | 1.65    | 1.92E-03      | 9.21E-03       | 0.73           |                    |
| 45  | Androsterone sulfate                          | Sulfated steroids                                 | Lipids                                    | 1.05    | 1.33E-02      | 3.81E-02       | 0.72           |                    |
| 46  | PC(36:2)                                      | Phosphatidylcholine                               | Lipids                                    | 2.64    | 4.01E-07      | 1.42E-05       | 0.71           |                    |
| 47  | PE(36:2)                                      | Phosphatidylethanolamine                          | Lipids                                    | 1.91    | 6.53E-05      | 6.89E-04       | 0.70           |                    |
| 48  | SM C18:1                                      | Phosphatidylglycolipid                            | Lipids                                    | 1.97    | 2.54E-04      | 1.99E-03       | 0.69           |                    |
| 49  | LysoPC(C22:0)                                 | Glycerophosphocholine                             | Lipids                                    | 2.34    | 3.45E-08      | 3.07E-06       | 0.69           |                    |
| 50  | L-Hexanoylcarnitine                           | Fatty acid esters                                 | Lipids                                    | 1.70    | 1.96E-02      | 4.96E-02       | 0.69           |                    |
| 51  | LysoPC(C14:0)                                 | Glycerophosphocholine                             | Lipids                                    | 1.19    | 7.82E-04      | 4.62E-03       | 0.68           |                    |
| 52  | Trigonelline                                  | Nicotinate and nicotinamide metabolism            | Energy metabolism                        | 2.28    | 3.96E-07      | 1.41E-05       | 0.67           |                    |
| 53  | Quinolinate                                   | Tryptophan metabolism                             | Amino acid                                | 1.52    | 8.70E-03      | 2.79E-02       | 0.67           |                    |
| 54  | 6-Hydroxynicotinate                           | Nicotinate and nicotinamide metabolism            | Energy metabolism                        | 1.68    | 5.78E-05      | 6.26E-04       | 0.66           |                    |
| 55  | PC(34:4)                                      | Phosphatidylcholine                               | Lipids                                    | 1.40    | 7.58E-03      | 2.53E-02       | 0.65           |                    |
| 56  | L-Tryptophan                                  | Tryptophan metabolism                             | Amino acid                                | 3.04    | 1.02E-12      | 7.8E-10        | 0.64           |                    |
| 57  | 3-Indoleacetonitrile                          | Tryptophan metabolism                             | Amino acid                                | 1.72    | 5.38E-04      | 3.47E-03       | 0.64           |                    |
| 58  | Xanthine                                      | Purine metabolism                                 | Nucleosides and related                   | 2.57    | 1.73E-07      | 9.05E-06       | 0.64           |                    |
| 59  | NAM                                           | Nicotinate and nicotinamide metabolism            | Energy metabolism                        | 2.66    | 1.12E-08      | 1.25E-06       | 0.60           |                    |
| 60  | Dodecanoic acid                               | Fatty acid metabolism                             | Lipids                                    | 1.43    | 1.65E-04      | 1.43E-03       | 0.60           |                    |
| 61  | UDP-D-xylene                                  | Nucleotide sugar metabolism                      | Carbohydrates and related                 | 2.14    | 2.62E-05      | 3.34E-04       | 0.58           |                    |
| 62  | 3-Hydroxy-L-kynurenine                        | Tryptophan metabolism                             | Amino acid                                | 2.08    | 1.09E-04      | 1.03E-03       | 0.58           |                    |
| 63  | 2-Aminoumuconic acid                          | Tryptophan metabolism                             | Amino acid                                | 1.34    | 6.98E-03      | 2.38E-02       | 0.56           |                    |
| 64  | PC(28:0)                                      | Phosphatidylcholine                               | Lipids                                    | 1.73    | 3.16E-03      | 1.34E-02       | 0.56           |                    |
| 65  | Sphinganine                                   | Sphingolipid metabolism                           | Lipids                                    | 2.71    | 1.16E-07      | 6.99E-06       | 0.52           |                    |
| 66  | PE(38:6)                                      | Phosphatidylethanolamine                          | Lipids                                    | 2.00    | 8.72E-04      | 5.03E-03       | 0.50           |                    |
| 67  | Apigenin 8-C-glucoside                        | Flavonoid biosynthesis                            | Lipids                                    | 2.62    | 3.18E-05      | 3.89E-04       | 0.49           |                    |
| 68  | PC(38:6)                                      | Phosphatidylcholine                               | Lipids                                    | 2.70    | 5.34E-07      | 1.75E-05       | 0.39           |                    |
| 69  | L-Kynurenine                                  | Tryptophan metabolism                             | Amino acid                                | 2.61    | 6.35E-06      | 1.11E-04       | 0.33           |                    |

Bold values indicate metabolites differentially expressed in both discovery and internal validation sets.
| ID | Metabolite name         | Subpathway                          | Superpathway             | VIP   | \( P \)-VALUE     | FDR   | Fold Change |
|----|------------------------|-------------------------------------|--------------------------|-------|-------------------|-------|-------------|
| 1  | Indolepyruvate         | Tryptophan metabolism               | Amino acid               | 1.95  | 2.13E-03          | 9.05E-03 | 3.68        |
| 2  | PC(33:4)               | Phosphatidylcholine                  | Lipids                   | 2.12  | 1.47E-05          | 2.41E-04 | 3.62        |
| 3  | Acetate                | Glycolysis / Gluconeogenesis         | Carbohydrates and related| 1.72  | 3.46E-03          | 1.30E-02 | 3.60        |
| 4  | ADPR                   | Nicotinate and nicotinamide metabolism| Energy metabolism       | 2.48  | 5.88E-04          | 3.53E-03 | 3.07        |
| 5  | Indole-3-acetate       | Tryptophan metabolism               | Amino acid               | 1.48  | 2.81E-04          | 2.03E-03 | 2.69        |
| 6  | PC(34:2)               | Phosphatidylcholine                  | Lipids                   | 2.27  | 2.86E-04          | 2.05E-03 | 2.69        |
| 7  | (S)-Lactate            | Glycolysis                           | Carbohydrates and related| 1.69  | 7.77E-08          | 9.67E-06 | 2.62        |
| 8  | Tryptamine             | Tryptophan metabolism               | Amino acid               | 1.26  | 3.50E-03          | 1.31E-02 | 2.52        |
| 9  | Deoxycholic acid       | Bile acids and derivatives           | Lipids                   | 1.14  | 1.65E-04          | 1.39E-03 | 2.47        |
| 10 | Phenylalanyl-Methionine| Methionine metabolism               | Amino acid               | 1.18  | 6.34E-04          | 3.74E-03 | 2.40        |
| 11 | Glycyl-Valine          | Valine metabolism                    | Amino acid               | 1.77  | 1.56E-04          | 1.33E-03 | 2.26        |
| 12 | N-Acetylhistidine      | Histidine metabolism                 | Amino acid               | 1.32  | 1.96E-02          | 4.51E-02 | 2.14        |
| 13 | Succinic acid semialdehyde| Fatty acid metabolism              | Lipids                   | 1.08  | 3.07E-04          | 2.16E-03 | 2.14        |
| 14 | D-ribose               | Pentose phosphate                    | Carbohydrates and related| 1.76  | 2.88E-04          | 2.06E-03 | 2.00        |
| 15 | Hydroxymethyldeoxyctydylate| Pyrimidine metabolism               | Nucleosides and analogues | 1.66  | 1.54E-06          | 4.92E-05 | 1.95        |
| 16 | Carnosine              | Histidine metabolism                 | Amino acids and analogues| 1.94  | 5.18E-07          | 2.40E-05 | 1.94        |
| 17 | Vitamin A              | Retinol metabolism                   | Vitamins                 | 1.89  | 1.09E-05          | 1.91E-04 | 1.90        |
| 18 | Asparaginyl-Valine     | Valine metabolism                    | Amino acid               | 1.77  | 1.27E-04          | 1.14E-03 | 1.82        |
| 19 | 1-MNAM                 | Nicotinate and nicotinamide metabolism| Energy metabolism       | 2.58  | 5.65E-07          | 2.55E-05 | 1.79        |
| 20 | L-Methylinosine        | Purine metabolism                    | Nucleosides and analogues| 1.71  | 3.57E-03          | 1.33E-02 | 1.79        |
| 21 | Formiminoglutaric acid | Histidine metabolism                 | Amino acid               | 1.44  | 4.86E-03          | 1.65E-02 | 1.71        |
| 22 | bicyclo-PGE2           | Eicosanoids                          | Lipids                   | 1.16  | 8.39E-03          | 2.46E-02 | 1.70        |
| 23 | Deoxyinosine           | Purine metabolism                    | Nucleosides and related  | 1.53  | 4.43E-03          | 1.54E-02 | 1.67        |
| 24 | L-lysine               | Lysine metabolism                    | Amino acid               | 1.96  | 3.97E-08          | 7.35E-06 | 1.66        |
| 25 | Aminoadipic acid       | Lysine biosynthesis                  | Organic acids and derivatives | 1.31  | 1.82E-02          | 4.29E-02 | 1.65        |
| 26 | D-Glucuronic acid      | Pentose and glucuronate interconversions| Carbohydrates and related| 1.92  | 6.51E-03          | 2.06E-02 | 1.64        |
| 27 | 8-Methoxykynurenate    | 8-Methoxykynurenate                  | Lipids and lipid-like molecules | 2.17  | 1.83E-05          | 2.83E-04 | 1.64        |
| 28 | Pyruvic acid           | Glycolysis / Gluconeogenesis         | Carbohydrates and related| 1.79  | 2.22E-04          | 1.72E-03 | 1.63        |
| 29 | LysoPC(C18:3)          | Glycerophosphocholines               | Lipids                   | 1.75  | 1.24E-03          | 5.99E-03 | 1.63        |
| 30 | Hippuric acid          | Phenylalanine metabolism             | Amino acids and analogues| 1.22  | 1.50E-02          | 3.77E-02 | 1.60        |
| 31 | Phenylalanine          | Phenylalanine metabolism             | Amino acids and analogues| 1.66  | 2.37E-04          | 1.80E-03 | 1.58        |
| 32 | Phenylalanyl-Isoleucine| Isoleucine metabolism                | Amino acid               | 1.80  | 3.67E-05          | 4.57E-04 | 1.57        |
| 33 | PE(38:4)               | Phosphatidylethanolamine             | Lipids                   | 2.23  | 2.64E-04          | 1.94E-03 | 1.57        |
| 34 | Trimethylamine N-oxide | Methanol metabolism                  | Organic nitrogen          | 1.23  | 5.09E-03          | 1.71E-02 | 1.55        |
| No. | Compound                        | Metabolism or Function                          | Energy metabolism       |
|-----|---------------------------------|------------------------------------------------|-------------------------|
| 35  | Propionic acid                  | Nicotinate and nicotinamide metabolism          |                         |
| 36  | D-Ribose 5-phosphate            | Pentose phosphate                               |                         |
| 37  | L-Serine                        | Serine metabolism                               | 1.54                    |
| 38  | Citrulline                      | Arginine biosynthesis                           | 3.74E-05                |
| 39  | L-Asparagine                    | Asparagine metabolism                           | 4.63E-04                |
| 40  | 5’-Methylthioadenosine          | Cysteine and methionine metabolism              |                         |
| 41  | 5-carboxamide                   | Nicotinate and nicotinamide metabolism          |                         |
| 42  | PC(42:2)                        | Phosphatidylcholine                             | 1.97                    |
| 43  | PC(40:7)                        | Phosphatidylcholine                             | 1.34E-02                |
| 44  | L-Methionine                    | Methionine metabolism                           | 3.84E-04                |
| 45  | Valyl-Lysine                    | Valine and lysine metabolism                    | 1.50                    |
| 46  | PC(34:1)                        | Phosphatidylcholine                             |                         |
| 47  | PC(33:3)                        | Phosphatidylcholine                             | 1.97                    |
| 48  | Orotidine                       | Pyrimidine metabolism                           |                         |
| 49  | Glutamyl-valine                 | Valine metabolism                               |                         |
| 50  | 3-Phospho-D-glycerate           | Pentose phosphate                               |                         |
| 51  | L-Acetylcarnitine               | Fatty acid metabolism                           | 1.30                    |
| 52  | Serine                          | Serine metabolism                               | 1.18                    |
| 53  | L-glutamic acid                 | Arginine metabolism                             | 1.25                    |
| 54  | PA(34:1)                        | Phosphatidic acid                               |                         |
| 55  | Histidinyl-Tryptophan           | Tryptophan metabolism                           |                         |
| 56  | Cysteine-S-sulfate              | Cysteine and methionine metabolism              |                         |
| 57  | Nicotinamide riboside           | Nicotinate and nicotinamide metabolism          |                         |
| 58  | PC(38:4)                        | Phosphatidylcholine                             | 2.10                    |
| 59  | L-Fucose                        | Fructose metabolism                             | 2.60E-05                |
| 60  | PC(34:3)                        | Phosphatidylcholine                             |                         |
| 61  | PE(34:1)                        | Phosphatidylethanolamine                        |                         |
| 62  | PC(36:2)                        | Phosphatidylcholine                             | 2.49                    |
| 63  | Heptadecanoyl carnitine         | Fatty acid metabolism                           | 3.44E-07                |
| 64  | Glycolic acid                   | Glyoxylate and dicarboxylate metabolism         |                         |
| 65  | PC(41:3)                        | Phosphatidylcholine                             | 1.01                    |
| 66  | PE(38:5)                        | Phosphatidylethanolamine                        |                         |
| 67  | Trigonelline                    | Nicotinate and nicotinamide metabolism          |                         |
| 68  | L-Tryptophan                    | Tryptophan metabolism                           | 1.71E-05                |
| 69  | 9-octadecenoic acid             | Lineolic acid metabolism                        | 2.69E-04                |
| 70  | 9-HODE                          | Lineolic acid metabolism                        |                         |
| 71  | 6-Hydroxyxicotinate             | Nicotinate and nicotinamide metabolism          |                         |
| 72  | Decanoic acid                   | Fatty acid biosynthesis                         |                         |
| 73  | Xanthine                        | Purine metabolism                               |                         |
| 74  | Nicotinate                      | Nicotinate and nicotinamide metabolism          |                         |
| 75  | PC(42:10)                      | Phosphatidylcholine                             | 3.91E-06                |
| 76  | L-taurine                       | Taurine metabolism                              | 8.85E-05                |

**Notes:**
- **Energy metabolism** values are given in units of energy units per unit of substrate.
- The table lists various substrates and their corresponding metabolism pathways, along with associated energy values.
| #  | Metabolite          | Pathway                          | Class      | 
|----|---------------------|----------------------------------|------------|
| 77 | NAM                 | Nicotinate and nicotinamide metabolism | Energy metabolism | 2.87 | 2.16E-06 | 6.04E-05 | 0.62 |
| 78 | Malonic acid        | Fatty acid metabolism            | Lipids     | 1.40 | 5.88E-05 | 6.41E-04 | 0.61 |
| 79 | PC(42:9)            | Phosphatidylcholine              | Lipids     | 1.97 | 7.44E-03 | 2.27E-02 | 0.60 |
| 80 | 3-Indoleacetonitrile| Tryptophan metabolism            | Amino acid | 1.86 | 6.57E-03 | 2.07E-02 | 0.59 |
| 81 | SM C18:1            | Phosphosphingolipids             | Lipids     | 2.64 | 1.12E-06 | 4.05E-05 | 0.59 |
| 82 | Jasmonic acid       | Linolenic acid metabolism        | Lipids     | 1.84 | 2.52E-04 | 1.88E-03 | 0.57 |
| 83 | 3-Hydroxy-L-kynurenine| Tryptophan metabolism            | Amino acid | 1.48 | 8.69E-03 | 2.52E-02 | 0.48 |
| 84 | PC(38:6)            | Phosphatidylcholine              | Lipids     | 2.20 | 3.18E-08 | 6.81E-06 | 0.42 |
| 85 | Quinolinate         | Tryptophan metabolism            | Amino acid | 1.89 | 2.03E-04 | 1.61E-03 | 0.42 |
| 86 | L-Kynurenine        | Tryptophan metabolism            | Amino acid | 2.20 | 2.32E-07 | 1.35E-05 | 0.36 |
| 87 | PE(38:6)            | Phosphatidylethanolamine         | Lipids     | 2.11 | 5.32E-04 | 3.27E-03 | 0.31 |
| 88 | Theophylline        | Purine derivatives               | Nucleosides and related | 1.50 | 3.92E-03 | 1.41E-02 | 0.26 |
| 89 | Theobromine         | Purine derivatives               | Nucleosides and related | 1.10 | 6.16E-03 | 1.98E-02 | 0.26 |

Bold values indicate metabolites differentially expressed in both discovery and internal validation sets.
Table S6
The additional values of the RF-based 6-metabolite model beyond the FREEDOM clinical risk score in the external validation set.

| FREEDOM risk score | FREEDOM risk score + 6-metabolite panel | Reclassified (%) |
|--------------------|----------------------------------------|------------------|
|                    | < 30% | 30-60% | > 60% | Total |
| Patients not occurring MACEs, No. | | | | |
| < 30% risk         | 187   | 27     | 1     | 215   | 13   |
| 30-60% risk        | 27    | 0      | 10    | 37    | 100  |
| > 60% risk         | 1     | 0      | 1     | 2     | 50   |
| Total              | 215   | 27     | 12    | 26    |
| Patients occurring MACEs, No. | | | | |
| < 30% risk         | 11    | 14     | 1     | 26    | 58   |
| 30-60% risk        | 0     | 4      | 15    | 19    | 79   |
| > 60% risk         | 0     | 0      | 2     | 2     | 0    |
| Total              | 11    | 18     | 18    | 64    |
| Combined group     | | | | |
| < 30% risk         | 198   | 41     | 2     | 241   | 18   |
| 30-60% risk        | 27    | 4      | 25    | 56    | 93   |
| > 60% risk         | 1     | 0      | 3     | 4     | 25   |
| Total              | 226   | 45     | 30    | 32    | |

Estimate    | $p$ value  |
|------------|------------|
| $\Delta$C-index | $0.20 \ (0.12-0.28)$ | $< 0.001$   |
| NRI Categorical | $0.60 \ (0.45-0.75)$ | $< 0.001$   |
| NRI Continuous | $1.35 \ (1.14-1.55)$ | $< 0.001$   |
| IDI          | $0.27 \ (0.22-0.32)$ | $< 0.001$   |

NRI: net reclassification improvement; IDI: integrated discrimination improvement.
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