Preparation protocols for M1-M6

In order to achieve accurate quantification for each metabolite in the samples, precise proportions were employed when preparing the mixed stock solutions, taking into consideration their practical amounts in serum.

M1: for amine acids and nucleosides

Accurately transfer 50 μL of serum samples, QCs or different concentration of standards to the 750 μL 96-well sample collection plates (Waters, Milford, USA). Subsequently, add 10 μL of mixed internal standard (M1-IS) and 190 μL of methanol to the serum samples and vortex shake at 1500 rpm for 3 min. Next, the mixture was centrifugated at 5300 rpm for 20 min under 4 ℃ and 180 μL of supernatant was transferred, with a total of 2 μL injected for LC-MS/MS analysis.

M2: for bile acids and fatty acids

Briefly, 50 μL of serum samples, QCs or standards were firstly combined with 180 μL of the extraction reagent (including methanol and acetonitrile, V: V=1: 1) into the sample collection plates. Then, 20 μL of mixed internal standard (M2-IS) was added and the mixture performed vortex shaking at 1500 rpm for 3 min, followed by centrifugation at 5300 rpm for 20 min. Afterward, 180 μL of the supernatant was transferred to the microplates for analysis (3 μL).

M3: for organic acids and carbohydrates

The analysis of organic acids employs derivatization strategies. In brief, 20 μL of samples were pretreated with 80 μL mixture of acetonitrile and methanol (1:1), followed by a short vortex and centrifugation at 12000 rpm for 20 min to precipitate the protein. Then, 40 μL of the supernatant was transferred into another tube, and 5 μL of M3-IS, 20 μL of the derivatization reagent 1 (3-NPH, 200 mM in 50% acetonitrile-water solution) and 20 μL of reagent 2 (EDC, 240 mM in 50% acetonitrile-water solution with 6% pyridine in it) was added together into the bottom. The obtained system was reacted at 40 ℃ for 30 min after vertex shaking and slight centrifugation. After that, the mixture was placed immediately under -20℃ to terminate the reaction and dried under vacuum for 30 min. Then, the residue was redissolved in 800 μL of 50% methanol-water solution and 2 μL of supernatant was injected.

M4: for bacteria-derived metabolites

To determine the levels of bacteria-derived metabolites, 50 μL of samples or standards were firstly transferred into the 750 μL 96-well sample collection plates. Then, 190 μL of the extraction reagent and 10 μL of M4-IS was added,

followed by vertex at 1500 rpm for 3 min. Then, the mixture in the 96-well plate was centrifuged at 5300 rpm for 20 min and both 120 μL of the supernatant and ultra-pure water were transferred to the 450 μL Nunc™ microWell™ 96-Well microplates. Finally, 200 μL of the supernatant was transferred to the microplates for analysis (5 μL).

M5: for acyl carnitines and lysophospholipids

50 μL of samples or standards, 50 μL of M5-IS and 150 μL of the extraction reagent methanol was transferred and added into the 750 μL 96-well sample collection plates. After vertex at 1500 rpm for 3 min and centrifugated at 5300 rpm for 20 min under 4 ℃, 180 μL of the supernatant was transferred to the 96-Well microplates (injection volume: 5 μL).

M6: lipids

Accurately transfer 20 μL of serum samples or QC samples to the corresponding centrifuge tube, followed by adding 120 μL of methanol containing mixed M6-IS and vortex at 1500 rpm for 3 min. Subsequently, 100 μL of water and 360 μL of MTBE were added and vortex at 1500 rpm for 5 min. After placed at 4 ℃ for 3 min, the mixture was centrifugated at 3000 rpm for 5 min, in which 300 μL of upper layer was transferred. Then, 360 μL MTBE was additionally added to the residue, and repeat the extracted operation, followed by the upper layer transferred to the same tube or plate. After dried under vacuum for 1h, 80 μL of isopropanol- acetonitrile-hexane (5/5/2, V/V/V) was used as the redissolved solution. Finally, 2 μL of supernatant was injected for analysis.