Supplemental methodology for metabolomic and proteomic analysis

Plasma and bile metabolomic analysis

Metabolite extraction

Samples were thawed at 4°C. 400μL extraction buffer (acetonitrile/methanol/acetone, 8:1:1 ratio) mixture was added to 50μL samples, then vortexed. The mixture was placed on ice for 30 minutes to precipitate proteins and then centrifuged at 13,000 rcf for 20 minutes at 4°C. 375μL supernatant was transferred to a new tube and split into two volumes: 187.5μL for positive mode LC-MS and 187.5μL for negative mode LC-MS. Metabolites were dried using a Thermo Scientific Savant SNA120 SpeedVac Concentrator and stored at -80°C until LC-MS analysis. Pooled samples were prepared for quality control (QC), which underwent the same sample preparation and analysis as test samples. A pool sample was used for each batch (4).

LC-MS/MS analysis

Chromatographic separation was performed on a Dionex Ultimate 3000 HPLC System (Thermo Fisher Scientific, Massachusetts, USA) with a Hypersil Gold C18 column (150 × 2.1 mm, 1.9 μm; Thermo Fisher Scientific) under gradient conditions, using mobile phases A (0.1% formic acid in 2% acetonitrile: water, v/v) and B (0.1% formic acid in 90% acetonitrile: water, v/v). Flow rate was set at 200 μL/min and the column was maintained at 40°C. Extracted metabolites were dissolved in 20 μL solvent A and 5 μL of sample was injected into the column for each run. The gradient started with 1% of solvent B and increased to 90% B in 17 minutes, holding at 90% B for 4 minutes, then 90% solvent B to wash the column for the next sample, and changed to 1% of B for 5 minutes to equilibrate the column. Mass spectrometry analyses were performed on an Orbitrap Fusion instrument (Thermo Fisher Scientific, Massachusetts, USA) fitted with a Heated Electrospray Ionization (HESI) source. Data were acquired in positive and negative modes for each LC-MS analysis. Source parameters for positive ionization mode were set as follows: spray voltage at +4.2 kV, sheath gas at 35 (arbitrary units), auxiliary gas at 10 (arbitrary units) and sweep gas at 2 (arbitrary units), while for negative ionization mode spray voltage - 3.2 kV, sheath gas 40, auxiliary gas 15 and sweep gas 1. The Ion transfer tube temperature was set at 300°C, and vaporizer temperature at 300°C. For MS1 acquisition, the data were acquired using the Orbitrap analyser, at a resolution of 120,000 at m/z 200 (FWHM, a spectrum collected at this resolution requires ~0.6 s); maximum injection time was set at 250 ms, automatic gain control (AGC) target at 5.0 x 104, RF lens level at 60% and the scan range from m/z 70-1000. The MS2 acquisition was performed in Orbitrap with resolution was 30,000 at m/z 200, isolation window of 1.0, scan range “normal”, maximum injection time 35 ms. The selected ions were sequentially fragmented by higher energy collisional dissociation (HCD) with stepped collision energy of 20%, 30% and 40%. In all cases, one microscan was recorded using dynamic exclusion of 3 seconds and mass tolerance of ±5ppm. A targeted exclusion list of background noise with mass tolerance of ±5ppm was also included in the method to remove the LC-MS contaminants.

LC-MS/MS data processing and database searching

Raw LC-MS metabolomics data were processed using MS-DIAL5 Desktop Application[1], using ESI(+)-MS/MS from standards+bio+in silico (16,746 unique compounds) for positive mode data and ESI(-)-MS/MS from standards+bio+in silico (15,100 unique compounds) for Negative mode data[2]. These databases were accessed/downloaded on February 13, 2024. MS-DIAL5 parameters were set as follows: MS1 centroid tolerance, 0.01Da; minimum peak height, 1,000; preferred ions, [M+H]+1; [M-H]-1; maximum retention time shift, 0.1 min; MS2

centroid mass tolerance of 0.025Da for library search. Background compounds were removed if the sample to blank area ratio was less than 5. Features identified with mass difference >5ppm between the observed mass of a compound and its annotated mass, and compounds with “not the top hit” or “partial match” or “no match” or “invalid mass”, were also filtered out. Filtered compounds were manually validated by matching fragmentation spectra to those of available standards or to spectra reported in the literature.

Plasma proteomic analysis

Protein extraction and digestion

Protein quantification was performed by Bradford assay. Equal amounts of protein (300μg) from the pooled QC and plasma samples were treated with Top14 Abundant Protein Depletion Mini Spin Columns (A36370, Thermo Fisher Scientific, Massachusetts, USA) according to manufacturer’s instructions. The depleted samples were precipitated by 1:6 acetone precipitation overnight at -20°C. The precipitated samples were centrifuged for 15 minutes at 13,000 rcf at 4°C, and the supernatant removed. The resulting pellets were dissolved in 8M urea and denatured with 50mM dithiothreitol by incubation at 60°C for 10 minutes. Samples were alkylated with 100mM iodoacetamide in the dark at room temperature for 30 minutes. The urea concentration was diluted to 2M by addition of 50mM ammonium bicarbonate buffer. Trypsin digestion with mass spectrometry-grade proteases (#90058, Thermo Fisher Scientific, Massachusetts, USA), was then performed at 37°C: first at 1:30 mass ratio (trypsin: protein) for 2 hours, then at 1:60 ratio overnight. An end concentration of 1% formic acid was added to quench the enzymatic activation of trypsin, and drop in pH confirmed. The peptide mixture was desalted using Bond Elut C18 columns (#12102001, Agilent Technologies, California, USA), dried using a Thermo Scientific Savant DNA120 SpeedVac Concentrator and stored at -80°C until LC-MS analysis.

LC-MS/MS analysis

The peptide mixture was dissolved in 20 µL buffer A (2% acetonitrile: 98% water with 0.1% formic acid) and separated by Dionex Ultimate 3000 HPLC System connected to a Thermo Orbitrap Fusion mass spectrometer equipped with an online Nanospray Flex™ Ion Source. 4µL peptide sample was loaded onto Thermo Trap Cartridge (PepMap™ Neo, C18, 100 Å, 300 µm x 5 mm) and eluted from analytical column (bioZen 2.6 µm Peptide XB-C18, Nano Column, 250 x 0.075 mm, Phenomenex Ltd, UK.) by using a 120-minute linear gradient from 5% to 30% of solvent B (90% acetonitrile: 10% water with 0.1% formic acid), followed by column wash and calibration at a flow rate of 400 nL/minute.
The Orbitrap Fusion mass spectrometer was operated in the data-dependent acquisition (DDA) mode. The MS1 survey scan of parent ion was from 350–1500 m/z, and data were acquired at a high resolution of 120,000 (m/z 200), the AGC target was set to 3×10^5 and the maximum injection time was 100ms. The second stage of mass spectrometry (MS2) scans was performed on IonTrap at rapid scan rate, with dynamic exclusion, 50 seconds; cycle time, 3 seconds; isolation width, 0.7 m/z and MIPS mode as peptide. Ions with charge states 2-7 were sequentially fragmented by collision induced dissociation (CID) with a fixed collision energy of 35%. For the setting of the Nanospray Flex ion source, the spray voltage of 2.2 kV was applied on Thermo Scientific Nano Bore steel emitter and ion transfer tube temperature was set at 275°C.

Tissue proteomic analysis

Protein extraction, digestion and sample multiplexing

500μL lysis buffer (0.5 % SDS; 0.1 M TEAB; Halt™ protease/phosphatase inhibitor cocktail (#78440 Thermo)) was added to each sample. Bead homogenisation was performed with Precellys Lysing kits (CK14 2ml) on the Precellys Evolution instrument at 6,500 rpm for 2 sets of 30 seconds with a 15-second break. The mixture was centrifuged at 4 °C for 10 minutes at 13,000 rpm. The pelleted cellular debris was discarded and the supernatant was transferred to a new centrifuge tube on ice. Protein concentration was determined by Bradford assay.
Approximately 100 µg of protein from each sample was reduced with 0.5 M TCEP for 1 hour at 60°C and alkylated with 40 mM iodoacetamide for 30 min at room temperature in the dark. After 10-fold dilution using 0.1 M TEAB, protein samples were digested at 37 °C using Pierce Trypsin Protease, MS Grade at 1:30 mass ratio (trypsin: protein) for 2 hours followed by a second step of trypsin at a mass ratio of 1:60 (trypsin: protein) overnight. The reaction was quenched with formic acid. Trypsin enzymatic peptides were dried under vacuum after desalination by SPE Cartridge Bond Elut C18 columns (Agilent Technologies, Santa Clara, USA). The peptides were dissolved with 0.1 M TEAB (Sigma Aldrich, UK) and labelled with isobaric mass tags according to the manufacturer instructions of the 11plex Tandem mass tag (TMT) kit (Thermo Fisher Scientific, UK). A pool sample was used to control batch effects between sets of TMT labelling.

LC-MS/MS analysis

TMT-labelled peptides were pre-fractionated in order to reduce sample complexity using a 150 mm C18 column on an Agilent 1260 Infinity II high performance liquid chromatography instrument (Agilent Technologies, USA). Gradient elution was used to collect 27 fractions in 60 min, using a linear gradient of 2% to 35% acetonitrile at pH 9. These fractions were then dried under vacuum, using a Thermo Scientific Savant DNA120 SpeedVac Concentrator and stored at -80°C until LC-MS analysis.
Dried TMT-labelled peptides were resuspended in 20 µL buffer A (2% acetonitrile: 98% water with 0.1% formic acid) and 5µl of sample was loaded onto Thermo Trap Cartridge (PepMap™ Neo, C18, 100 Å, 300 µm x 5 mm) and eluted from analytical column (bioZen 2.6 µm Peptide XB-C18, Nano Column, 250 x 0.075 mm, Phenomenex Ltd, UK). Online separation was performed using a Dionex Ultimate 3000-high performance liquid phase system, by eluting the peptides with a 65-minute linear gradient: 2–25% solvent B (90% ACN, 0.1% FA), 10 minutes; 25–50% solvent B, 5 minutes; 50–90% solvent B, 8 minutes; column wash with 90% solvent B, followed by 16 minutes column equilibration with solvent A at a flow rate of 400 nL/min.
MS analysis was performed using an Orbitrap Fusion mass spectrometer equipped with a Nanoflex ion source (Thermo Fisher Scientific, UK). The parent ions of the peptide were detected and analysed using a high resolution orbitrap with ion source voltage 2.2 kV. MS1 scan range was set at 375–1500 m/z, with resolution of 120,000 at m/z 200. MS2 was performed using an ion-trap at rapid scan rate, with the following parameters: charge state 2-7, dynamic exclusion of 50 seconds, cycle time 3 seconds and collision induced dissociation ~35%. Automated Synchronous Precursor Selection for MS3 was used for quantification of TMT11plex and performed on the orbitrap at 30,000 resolutions, with scan range 100-500 m/z, maximum injection time 105 ms and with 65% high-energy collisional dissociation.

<u>Protein LC-MS/MS data processing and database searching</u>

Protein identification was performed using the CHIMERYS 3.0, AI-powered search algorithm (MSAID Gmbh, Germany) and MASCOT search engine (version 2.3.2; Matrix Science, London, UK) embedded in Proteome Discoverer 2.2 (Thermo Fisher Scientific, Massachusetts, USA). The LC-MS/MS raw data were searched against the Sus scrofa database obtained from UniProt (version 2024_03_27 containing 292,143 protein sequences).

Proteome Discoverer parameters were set as follows: MS1 mass tolerance was set to 10ppm while MS2 mass tolerance was 0.6 Da for IonTrap detection, trypsin was chosen as the enzyme, carbamidomethylation of cystine was selected as fixed modification and oxidation of methionine, deamidation of asparagine and glutamine were specified as dynamic modifications. For the tissue proteomic analysis, in the Proteome Discoverer settings for TMT modifications, the TMT-11plex reagent was set as a fixed modification on lysine residues and peptide N-termini to ensure accurate quantification across samples. Only unique peptides were used for quantification of proteins. Only proteins with Mascot score ≥21 ($p<0.05$), at least one unique peptide and that unique peptide was identified with two peptide-spectrum matches (PSMs), were accepted and included in further analysis.

Statistical analysis

Porcine proteins were mapped to human genes using the UniProt mapping tool. Protein and metabolite abundance data were filtered to remove analytes present in less than 10% samples. For the plasma (but not tissue) studies, missing data were imputed using random forest. Batches were quantile normalised, batch effect corrected (ComBat algorithm) and log2 transformed. To calculate the differential abundance, an empirical bayes moderated t-statistic was calculated (LIMMA). For plasma metabolites and proteins, the treatment group: time interaction and effect of time for control samples were evaluated. For bile metabolites, bile samples pre-connection and at 16-hours were compared to perfusate and plasma samples at the corresponding time points. For tissue proteins, end-ELC samples were compared to pre-NMP samples. A false discovery rate <5% using the Benjamini–Hochberg method was considered significant.
Principal component analysis was performed as follows:

- Plasma proteomics and metabolomics: common proteins with p-value < 0.05 in both treatment group: time and control time analyses.
- Tissue proteomics: proteins with p-value < 0.05 in end-ELC vs pre-NMP analysis.
- Bile metabolomics: metabolites with p-value < 0.05 in 16-hour bile samples.

Volcano plots highlight species with adjusted p-value < 0.05 (< 0.01 in bile metabolome volcano plot) and absolute logFC > mean logFC + standard deviation as significant.
Heatmaps were produced from the top 50 differentially-expressed species as follows:

- Plasma proteomics: adjusted p-value < 0.05 in treatment group: time and/or control time analyses and p-value < 0.05 in the other analysis.
- Plasma metabolomics: adjusted p-value < 0.05 in treatment group: time or control time analyses.
- Tissue proteomics: adjusted p-value < 0.05 in end-ELC vs pre-NMP analysis.
- Bile metabolomics: adjusted p-value < 0.05 in bile at 16 hours vs plasma at 16 hours (upregulated metabolites only).

Proteomic pathway analysis was performed using Reactome[3]. Metabolomic pathway analysis was performed for metabolites with p-value < 0.05 using the SMPDB library on web-based tool MetaboAnalyst version 6.0[4]. R packages ggplot2 and pheatmap were used for graphical presentation.

References

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