Protocol for sample preparation, LC-MS/MS, and data analysis

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

For metabolomics analyses, cold acetonitrile was added to plasma samples (2:1, v/v) vortexed and centrifuged (10 min, 10000 rpm at 4 °C) for protein precipitation. Stable isotopes caffeine-$^{13}$C3, tyrosine-15N and progesterone-d9 were used as internal standards and samples were transferred to injecting vials for LC-MS/MS analysis, which was performed with High-Performance Liquid Chromatography (HPLC-UV, 1220 Infinity, Agilent Technologies) coupled with Q Exactive hybrid Quadrupole-Orbitrap high-resolution mass-spectrometer (Thermo Fisher). Reverse phase C18 chromatography was performed with Zorbax Eclipse Plus C18 column (4.6 x 150 mm 3.5 µm Agilent) and positive electrospray ionization. All samples were analyzed using a gradient elution program. The binary mobile phases were water 0.5% formic acid with 5 mM of ammonium formate (A), and acetonitrile (B). Their gradient elution started with 20% (B) for 5 min, then linearly increased to 100% (B) in 30 min and kept constant for 8 min in 100% (B). The eluent was restored to the initial conditions in 4 minutes to re-equilibrate the column and held for the remaining 8 minutes. The flow rate was kept at 0.5 mL min-1. The injection volume for analysis was 3 µL, and the column temperature was set at 35 °C. The electrospray ionization was operating with the following settings: spray voltage 3.5 kV; capillary temperature: 269 °C; S-lens RF level 50 V; sheath gas flow rate at 53 L min-1; aux gas flow rate at 14 L min-1; sweep gas flow rate 3 L min-1. The high-resolution mass-spectrometry was obtained under full MS/dd-MS2 mode. The mass range in the full MS scanning experiments was m/z 80-1200. The max IT was set at 200 ms, and AGC target was set at 1 x 106. For fragmentation acquisition, the top 5 (TopN, 5, loop count 5) most abundant precursors were sequentially transferred into the C-Trap (AGC target 1 x 105; max IT 50 ms) for collision. The collision energy for target analytes was 20, 30 and 35 eV. Resolving power was set at 140,000 and 70,000 for full MS and dd-MS2 acquisitions, respectively.

Bioinformatics and statistical analyses

Proteowizard software was used to convert .raw files into mzXML format and apLCMS software was used to perform peak deconvolution and detection, to filter noise, to align mass-to-charge ratio (m/z) and retention time and to quantify metabolite features, which are defined by a specific m/z, retention time and intensity values for each sample. Pooled human plasma samples were used as quality control (QC) and included in every batch of samples. Replicate samples were summarized based on Pearson correlation $r > 0.7$. Data were log2 transformed and features were filtered out by 90% presence in all samples and a coefficient of variation < 0.2 based on QC samples.