Reversed phase LC-MS and LC-MS/MS non-targeted lipidomics

Non-targeted profiling of lipid metabolites was conducted at the Georgia Institute of Technology.

Sample preparation

All solvents were LC-MS grade and were purchased from ThermoFisher Scientific. All stable isotope-labeled internal standards (IS) were purchased from Avanti Polar Lipids (Alabaster, Alabama): of PC (15:0-18:1(d7)); PE (15:0-18:1(d7)); PS (15:0-18:1(d7)); PG (15:0-18:1(d7)); PI (15:0-18:1(d7)); LPC (18:1(d7)); LPE (18:1(d7)); Chol Ester (18:1(d7)); DG(15:0-18:1(d7)); TG (15:0-18:1(d7)-15:0); SM (18:1(d9)); and cholesterol (d7). IS were added to the extraction solvent at a final concentration in the 0.1-8 µg/ml range.

Plasma samples: Samples were stored at -80 ºC until extraction. An ice bath was used to thaw and maintain temperature throughout preparation. Plasma samples (25 µL) were vortex mixed with 75 µL of the extraction solvent (isopropanol containing the IS mix listed above) followed by centrifugation for 5 min at 21,100 x G to pellet insoluble material. Supernatant was transferred to autosampler vial and stored at 4 ºC until analysis. An aliquot from each supernatant was combined to create a pooled sample used as a quality control (QC).

Tissue samples: Samples were stored at -80 ºC until extraction. An ice bath was used to thaw the samples. Powdered tissue samples (10 mg) with 400 µl of the extraction solvent (isopropanol containing IS mix listed above) were extracted by freeze-thawing in liquid nitrogen for 1 min followed by sonication in an ice bath for 3 min, repeated three times. Sample were vortex mixed for 5 min in pulsed mode followed by centrifugation for 5 min at 21,100 X G. Supernatant was transferred to autosampler vial and stored at 4 ºC until analysis. An aliquot from each supernatant was combined to create a pooled sample.

Sample blanks and consortium reference samples were prepared for analysis using the same methods.

LC-MS and LC-MS/MS analysis

Lipid LC-MS data were acquired using a Vanquish (ThermoFisher Scientific) chromatograph fitted with a ThermoFisher Scientific Accucore™ C30 column (2.1 × 150 mm, 2.6 µm particle size), coupled to a high-resolution accurate mass Q-Exactive HF Orbitrap mass spectrometer (ThermoFisher Scientific) for both positive and negative ionization modes. The mobile phases were 40:60 water:acetonitrile with 10 mM ammonium formate and 0.1% formic acid (mobile phase A) and 10:90 acetonitrile:isopropyl alcohol, with 10 mM ammonium formate and 0.1% formic acid (mobile phase B). The column temperature was set to 50°C, the injection volume was 2 µL, and the gradient program is shown in Table 5.

Table 5. LC-MS gradient program

Time (min)	%A	%B	Flow (ml/min)	Curve
0	80	20	0.4	5
1	40	60	0.4	6

5	30	70	0.4	6
8	10	90	0.4	6
8.2	0	100	0.4	6
10.5	0	100	0.4	6
10.7	80	20	0.4	6
12	80	20	0.4	6

For analysis the electrospray ionization source was operated at a vaporizer temperature of 425°C, a spray voltage of 3.0 kV for positive ionization mode and 2.8 kV for negative ionization mode, sheath, auxiliary, and sweep gas flows of 60, 18, and 4 (arbitrary units), respectively, and capillary temperature of 275°C. The instrument acquired full MS data with 240,000 resolution over the 150-2000 *m/z* range. Samples were analyzed in random order with pooled QC injections collected at minimum every tenth injection.

LC-MS/MS experiments were acquired using a data dependent acquisition (DDA) strategy to aid in compound identification. MS^2 spectra were collected with a resolution of 120,000 and the dd-MS^2 were collected at a resolution of 30,000 and an isolation window of 0.4 *m/z* with a loop count of top 7. Stepped normalized collision energies of 10%, 30%, and 50% fragmented selected precursors in the collision cell. Dynamic exclusion was set at 7 seconds and ions with charges greater than 2 were omitted.

Data processing and quality control

Feature detection and alignment: Data processing steps included peak detection, spectral alignment, and gap filling and were performed with Compound Discoverer V3.0 (ThermoFisher Scientific) to yield an aligned feature table containing *m/z*, RT, and relative peak areas.

Data cleaning and degeneracy removal: Compound Discoverer was used to group isotopic peaks as well as adduct ions to simplify the feature table. Detected features were filtered with background and QC filters. Features with abundance lower than 5x the background signal in the sample blanks and that were not present in at least 50% of the QC pooled injections with a coefficient of variance (CV) lower than 30% were removed from the dataset.

Drift correction: Drift correction was performed on each individual feature, where a linear curve was fitted to the pooled QC sample peak areas across the batch and was then used to correct the peak area for that specific feature in the samples.

Quality control: System suitability was assessed prior to the analysis of each batch. A performance baseline for a clean instrument was established before any experiments were conducted. The mass spectrometers were mass calibrated, mass accuracy and mass resolution were checked to be within manufacturer specifications, and signal-to-noise ratios for the suite of IS checked to be at least 75% of the clean baseline values. For LC-MS assays, an IS mix consisting of 12 standards was injected to establish baseline separation parameters for each new column. The performance of the LC gradient was assessed by inspection of the column back pressure trace, which had to be stable within an acceptable range (less than 30% change). Each IS mix component was visually evaluated for chromatographic peak shape, retention time (lower than 0.2 min drift from baseline values) and FWHM lower than 125% of the baseline

measurements. The CV of the average signal intensity and CV of the IS (<=15%) in pooled samples were also checked. These pooled QC samples were used to correct for instrument sensitivity drift over the various batches using a procedure similar to that described by the Human Serum Metabolome (HUSERMET) Consortium[42]. To evaluate the quality of the data for the samples themselves, the IS signals across the batch were monitored, PCA modeling for all samples and features before and after drift correction was conducted, and Pearson correlations calculated between each sample and the median of the QC samples.

Compound identification/annotation: Lipid annotations were accomplished based on accurate mass and relative isotopic abundances (to assign elemental formula), retention time (to assign lipid class), and MS^2 fragmentation pattern matching to local spectral databases built from curated experimental data. Lipid nomenclature followed that described by Fahy et al.[43,44]. When possible, features were matched to authentic compounds were identified with MSI level 1. Features that were matched to local databases were annotated with MSI level 2 or compound-class annotations with MSI level 3. Unknown features were assigned MSI level 4. Lipid annotations are highly subject to the available structural information to assign alkyl chain lengths, alkyl chain position, double bond position, and double bond stereochemistry. Annotations reflect the available structural information, which results in a feature with multiple possible lipid structures.