Metabolomic Analyses

Samples were stored at -80°C until analyzed via Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS and LC-HRMS/MS)-based metabolomics in the Vanderbilt Center for Innovative Technology (CIT) using previously described methods (PMID: 34068340 PMID: 27955696 PMID: 29774083). Briefly, cell pellets were normalized by total protein (200 ug) and the corresponding cell supernatants were normalized by volume (200uL). Metabolites were extracted with methanol/water 80:20. Heavy labeled phenylalanine-D8 and biotin-D2 were added to individual samples prior to protein precipitation. Following overnight incubation at -80°C, precipitated proteins were pelleted by centrifugation at 10,000 rpm for 10 min and metabolite extracts were dried down *in vacuo* and stored at -80°C.

Individual extracts were reconstituted in 100 µl of acetonitrile/water (3:97, v/v) with 0.1% formic acid containing heavy-labeled carnitine-D9, tryptophan-D3, valine-D8, and inosine-4N15, and centrifuged for 5 min at 10,000 rpm to remove insoluble material. A pooled quality control sample (QC) was prepared by pooling equal volumes of individual samples. The pooled QC sample was used for column conditioning (8 injections prior to sample analysis), retention time alignment and to assess mass spectrometry instrument reproducibility (1 injection every 5 sample injections) throughout the sample set and to determine sample acceptance.

Global, untargeted mass spectrometry analyses were performed on a high-resolution Q-Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Vanquish UHPLC binary system (Thermo Fisher Scientific, Bremen, Germany). Extracts (6µL injection volume) were separated on a Hypersil Gold, 1.9 µm, 2.1 mm × 100 mm column (Thermo Fisher) held at 40 °C. LC was performed at 250 µL/min using solvent A (0.1% FA in water) and solvent B (0.1% FA in acetonitrile/water 80:20) with a gradient length of 30 min as previously described (PMID: 33416496 PMID: 32566710). Full MS analyses were acquired over the mass-to-charge ratio (*m/z*) range of 70-1,050 in positive ion mode. Full mass scan was acquired at 120,000 resolution with a scan rate of 3.5 Hz, automatic gain control (AGC) target of $1x10^6$, and maximum ion injection time of 100 ms, and MS/MS spectra were collected at 15,000 resolution, AGC target of $2x10^5$ ions, with a maximum ion injection time of 100 ms.

Metabolomics Data Processing, and Pathway Analysis

Mass spectrometry raw data was imported, processed, normalized and reviewed using Progenesis QI v.3.0 (Non-linear Dynamics, Newcastle, UK). All MS and MS/MS sample runs were aligned against a pooled QC reference run. Unique ions (retention time and m/z pairs) were de-adducted and de-isotoped to generate unique "features" (retention time and m/z pairs). Data were normalized to all features and significance was assessed using p-values generated using ANOVA (analysis of variance) from normalized compound abundance data. Tentative and putative annotations were determined by using accurate mass measurements (< 5 ppm error), isotope distribution similarity, and fragmentation spectrum matching (when applicable) by searching the Human Metabolome Database (HMDB) (PMID: 23161693), METLIN (PMID: **16404815**), and the CIT's in-house library. Annotations (Confidence Level 1-3) (PMID: **27624161**) were determined for all compounds with a match to any of the searched libraries or databases.

Metaboanalyst 5.0 (www.metaboanalyst.ca/) was used to perform pathway and metabolite enrichment analyses from annotated compounds with statistical significance (p-value $\leq$ 0.05) (PMID: 31756036).