Metabolomics Analyses

Optima grade LC-MS solvents and chemicals for the mass spectrometry analyses were obtained from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA).

CSF samples collected were flash frozen and 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 (cite). Briefly, equal volumes (100 µL) of previously frozen CSF was diluted with 100 µL ice-cold lysis buffer (1:1:2, Acetonitrile:MeOH:Ammonium Bicarbonate 0.1M, pH 8.0, LC-MS grade). Addition of isotopically labeled phenylalanine-D8 and biotin-D2 were added to individual samples prior to protein precipitation by addition of 800 µL of ice-cold methanol. 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 until reconstitution prior to MS analysis.

Prior to mass spectrometry analysis, individual extracts were reconstituted in 60 µl of acetonitrile/ water (80:20, v/v) containing isotopically labeled standards, 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 CSF samples; the pooled QC sample was extracted in parallel with the individual samples. The pooled QC sample was used for column conditioning (10 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). CSF extracts (5uL injection volume) were separated on an ACQUITY UPLC BEH Amide HILIC 1.7µm, 2.1 × 100 mm column (Waters Corporation, Milford, MA) held at 30°C as previously described (cite). Briefly, liquid chromatography was performed at 200 µL min−1 using solvent A (5 mM ammonium formate in 90% water, 10% acetonitrile and 0.1% formic acid) and solvent B (5 mM ammonium formate in 90% acetonitrile, 10% water and 0.1% formic acid) with a gradient length of 30 min.

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 1x106, and maximum ion injection time of 100 ms, and MS/MS spectra were collected at 15,000 resolution, AGC target of 2x105 ions, with a maximum ion injection time of 100 ms.

Metabolomics Data Processing, Statistical 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. One-way repeated measure ANOVA was performed to examine CSF urea cycle metabolites in PRE participants and controls (PRE vs. control) for their baseline (BL) visits and follow up (FU) visits. Univariate ANOVA was performed to identify differences in urea cycle metabolites among controls, PRE, MAN, and LATE participants. Pearson correlations were used to determine correlations between CSF metabolites and CSF Neurofilament (NfL) and CAP-score.

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) (cite), METLIN (cite), and the CIT's in-house library. Annotations (Level 1-3) (cite) were determined for all significant compounds (p-value ≤ 0.05) with a match to any of the searched libraries or databases.

Metaboanalyst 4.0 (www.metaboanalyst.ca/) was used to perform pathway and metabolite enrichment analyses from annotated compounds with statistical significance (p-value ≤ 0.05) between BL and FU, genotype and disease stage (cite). Briefly, MetaboAnalyst assigns a pathway impact factor which reflects the location of an enriched compound within that particular metabolic pathway and its impact on the downstream targets. Negative log p-values were calculated based upon the number of observed compounds detected by LC-HRMS/MS within a particular metabolic pathway compared to the expected number of compounds known to be present in that pathway. The complete, de-identified raw data file for metabolomics can be found in Supplemental Material.