Metabolomics Preparation and Analysis

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Sample Preparation

1 AAV-PHP.S:hSYN1-hM3Dq-mRuby2 (1012VGs) was delivered systemically to TH-Cre and ChAT-Cre mice. 3-4 week after infection, C21 (3mg/kg) was administered daily for 10 consecutive days.

2 Fecal pellets were collected in sterile containers, and cecal contents upon sacrifice of the mice. Fecal and cecal samples were transported on dry ice for metabolomics analysis.

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4 The samples were weighed out and an extraction solvent (1:1 methanol to water with an internal standard of 1 µM sulfamethazine) was added at a 1:10 milligram to microliter ratio.

5 The samples were then homogenized using a TissueLyser II (Qiagen) for 5 minutes at 25 hertz followed by a 15 minute centrifugation at 14,000 rpm.

6 From the supernatant, 120 µL were transferred to a 96 deepwell plate (Eppendorf) and then these samples were lyophilized using a CentriVap Benchtop Vacuum Concentrator (Labconco) and stored at −80.

7 Upon the time for data acquisition, the lyophilized plates were resuspended in a 1:1 m$^{\circ}$Cethanol to water solvent spiked with 1 µM of sulfadimethoxine.

8 The plates were vortexed for 2 minutes, centrifuged at 14,000 rpm for 15 minutes and 120 µL of the supernatant was transferred to a 96 well autosampler plate (Eppendorf).

9 Plates were stored at 4 °C prior to LCMS analysis.

Data acquisition

The untargeted metabolomics analysis was completed using an ultra-high performance liquid

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10 chromatography system (Thermo Dionex Ultimate 3000 UHPLC) coupled to ultra-high resolution quadrupole time of flight (qTOF) mass spectrometer (Bruker Daltonics MaXis HD). Phenomenex Kinetex column (C18 1.7 µ 2.1 mm x 50 mm) was used for chromatographic separation.

11 An injection volume of 5 µL was used per sample and a flow-rate of 0.500 mL was used throughout the analysis. The mobile phase consisted of solvent A: 100% LC-MS grade water spiked with 0.1% formic acid and solvent B: 100% LC-MS grade acetonitrile spiked with 0.1% formic acid. The chromatographic gradient was as follows: 0.0–1.00 n, 5% B; 1.0– 9.00 n, 5–100% B; 9.0-11.00 n, 100% B; 11.0-11.50 n, 100-5% B; 11.5-12.5 min, 5% B. The data was collected using electrospray ionization in positive mode. Sample data was saved as .d file folders.

Data processing

12 The raw .d data files were converted to mzXML format using Bruker Compass DataAnalysis 4.1 software. The resulting .mzXML file, the original .d file folders, and basic prep information sheet were stored on the UC San Diego MassIVE data repository under the accession number MSV000084550.

13 For the MS1 level feature detection, the open-source software MZmine version 2.51 was used. The parameters used are as follows: 1) Mass Detection (Centroid, Noise Level MS1 1E3, MS2 1E2); 2) ADAP Chromatogram Builder (Min Group size in # of scans=3, Group Intensity Threshold= 3E3, Min Highest Intensity=1E3, m/z tolerance 0.01 m/z or 10.0 ppm); 3) Chromatogram Deconvolution (Local Minimum Search>Chromatographic Threshold 0.01%, Minimum in RT range 0.50 min, <Minimum Relative Height 0.01%, Minimum Absolute Height 3E3, Min Ratio of Peak Top/Edge 2, Peak Duration Range 0.05-0.50 min; m/z Calculation Auto, m/z range for MS2 pairing 0.01 Da, and RT Range for MS2 Pairing 0.1 min); Isotopic Peaks Grouper (m/z Tolerance 0.01 m/z or 10.0 ppm, Retention Time Tolerance 0.3 min, Maximum Charge 4, Representative Ion Most Intense); Join Aligner (m/z Tolerance 0.01 m/z or 10.0 ppm, Weight for m/z 75, Retention Time Tolerance 0.3 min, Weight for RT 25); Gap-Filling Peak Finder (Intensity Tolerance 20%, m/z Tolerance 0.005 m/z or 10.0 ppm, Retention Time Tolerance 0.2 min).

14 The resulting feature table was saved as a .csv file and .mgf file for use in GNPS and MetaboAnalyst.

Molecular networking and statistical analysis

15 Molecular networking was performing using the feature networking tool available on the Global Natural Products Social Molecular Networking portal (GNPS) accessed via the following link:https://gnps.ucsd.edu/ProteoSAFe/index.jsp?params=%7B%22workflow%22:%22FEATURE-BASED-MOLECULAR-NETWORKING%22,%22library_on_server%22:%22d.speclibs;%22%7D.

16 The annotations obtained using this workflow fall under MSI level 2 or 3 and were used for

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feature analysis (**Sumner et al., 2007**).

17 Briefly, level 2 compounds are putatively annotated meaning they are not identified using chemical reference standards but rather based on physical properties and/or spectral similarities to available spectral libraries (publicly available and purchased NIST17 CID).

18 Level 3 compounds are putatively characterized classes of compounds identified similarly to level 2 compounds. The feature based molecular networking workflow on GNPS (**Nothias et al., 2019**) was utilized in order to analyze the spectra associated with the feature tables produced using the open source software Mzmine version 2.51 (**Pluskal et al., 2010**). The .mgf and .csv outputs from MZmine v2.51 were used to run the workflow. The GNPS workflow parameters used we as follows: Precursor Ion Mass 0.02 Da, Fragment Ion Mass Tolerance 0.02 Da, Min Pairs Cos 0.7, Minimum Matched Fragments 6, Maximum Shift Between Precursors 500 Da, Network TopK 10, Maximum Connected Component Size (Beta) 100, and the files were row sum normalized. Default parameters were used for the rest of the settings.

19 The visualizations and statistical analyses were performed using QIIME 2 v.2019.10 (**Bolyen et al., 2019**), MetaboAnalyst and Cytoscape v3.7.2.

20 All PCoA axes in**Figure 6**and S5 were individually plotted in QIIME 2 and due to a batch effect between subsequent plates run on the mass spectrometer-axes that minimized experimental biases were determined and chosen for graphical representation.

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