**Nile Rat Plasma Metabolomics – fasting vs. random**

**Objective**

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Nile rats are a unique model system to explore incidence and biomarkers of diabetes because Nile rats have increased susceptibility to developing hyperglycemia when provided typical rodent chow diet during early development months (conception through weaning) vs. when provided a high-fiber rabbit chow diet in earlier development months. One of the challenges with Nile rats is that blood collections need to happen when animals are still quite young in order monitor diabetic progression and typical fasted-state blood collections could lead to increased stress in young animals. Random (non-fasted) blood sampling would be a better alternative; however, different postprandial states will likely drive higher metabolite variance in non-fasted samples.

This study aims to determine feasibility of random blood glucose-level sampling versus the standard fasted blood glucose sampling on diabetic Nile rats. The analysis presented here performed liquid chromatograph-mass spectrometry metabolomics on Nile rat plasma samples (same samples as report no. 0191, Nile Rat Plasma Lipidomics). Raw data were processed and subjected to preliminary data analysis.

**Results Summary**

**Combined\_metab\_lipid\_table.xlsx** contains the combined lipidomics and metabolomics data. Lipidomics data is identical to what was previously shared.

**Explanation of columns:**

**Unique ID:** unique identifier for each metabolite

**Type:** metabolite or lipid

**i:** shortened unique ID

**Proposed ID:** Current best estimate of metabolite ID. For any row marked as ‘metabolite’, then only IDs with Score > 80, or Score = ‘Tracefinder confirmed’ should be considered as confident IDs.

**Tracefinder ID:** Confident Identification from Tracefinder targeted metabolomics workflow.

**Lipid Class:** Same as previous report, gives class abbreviation for lipids.

**m/z:** Ion m/z used for quantification

**RT [min]:** Retention Time of metabolite peak

**Area (Max.):** log2-transformed area of the maximum found metabolite peak

**Score:** Confidence Score of metabolite Proposed ID for Compound Discoverer metabolites

**Adduct:** adduct of feature

**Polarity:** positive or negative

**Data Columns (60 total columns), e.g. 1076\_10\_FBG:** sample formatted as [Animal#]\_[week]\_[FBG/RBG]. All values are log2-transformed.

**p-value columns (6 total columns):**

3 columns are original p-values. 3 columns are FDR corrected. Recommend using FDR corrected p-values. Blank cells in p-value columns correlate with statistical models that fail to converge. See description of statistics in Data Analysis (below).

**Future Plans**

Ben will draft figures for manuscript. Ben will analyze data to determine ability of metabolite levels in predicting glucose tolerance and diabetic status. All parties will begin writing manuscript.

**Sample Preparation**

**Metabolomics sample preparation (same as report no. 0191):**

Plasma samples were removed from freezer and thawed on ice. Each sample was extracted with 500 uL 6:2:2 n-butanol:acetonitrile:water. Samples were vortexed for 10 s and then centrifuged at 14,000 x g for 2 min at 4 °C to precipitate protein. 100 uL of extract was dried down in an amber autosampler vial with glass insert by SpeedVac evaporator. For metabolomics, each extract was resuspended in 25 uL 1:1 Acetonitrile:Water then analyzed on the mass spectrometer.

**LC-MS Analysis**

**HILIC-LC-MS Metabolomics:** Sample analysis was performed on a ZIC-pHILIC HPLC column held at 50 °C (100 mm x 2.1 mm x 1.7 μm particle size; Millipore) using a Vanquish Binary Pump (150 μL/min flow rate; Thermo Scientific). Mobile phase A consisted of 10 mM ammonium acetate in ACN:H2O (10:90, v/v) containing 0.1% ammonium hydroxide. Mobile phase B consisted of 10 mM ammonium acetate in ACN:H2O (95:5, v/v) containing 0.1% ammonium hydroxide. Mobile phase B was initially held at 95% for 2 min and then decreased to 30% over 18 min. Mobile phase B was held for 6 min at 35%, then raised to 95% over 1 min. The column was re-equilibrated at 95% mobile phase B for 8 min. 2 µL of extract was injected by a Vanquish Split Sampler HT autosampler (Thermo Scientific).

The LC system was coupled to a Q Exactive-HF Orbitrap mass spectrometer through a heated electrospray ionization (HESI II) source (Thermo Scientific). Source conditions were as follow: HESI II and capillary temperature at 350 °C, sheath gas flow rate at 40 units, aux gas flow rate at 15 units, sweep gas flow rate at 1 units, spray voltage at |3.0 kV| for both positive and negative modes, and S-lens RF at 50.0 units. The MS was operated in a polarity switching mode acquiring positive and negative full MS and MS2 spectra (Top10) within the same injection. Acquisition parameters for full MS scans in both modes were 60,000 resolution, 1 × 10e6 automatic gain control (AGC) target, 100 ms ion accumulation time (max IT), and 70 to 900 m/z scan range. MS2 scans in both modes were then performed at 45,000 resolution, 1 × 10e5 AGC target, 100 ms max IT, 1.0 m/z isolation window, stepped normalized collision energy (NCE) at 20, 30, 40, and a 30.0 s dynamic exclusion.

**Data Analysis**

**Compound Discoverer 3.3 Metabolomics Data Processing and Analysis:** Data were analyzed starting from a default workflow (Untargeted Metabolomics using Online Databases, mzLogic, and Molecular Networks) with the following modifications:

1. Select Spectra
   1. Upper RT limit = 22
2. Align Retention Times (ChromAlign)
   1. Reference file = 20210831\_KAO\_HILIC\_T1082M\_20210401\_9wk\_RBG
3. Detect Compounds
   1. Mass Tolerance = 10 ppm
   2. Min. Peak Intensity = 50,000
   3. Ions = [M+FA-H]-1; [M+H]+1; [M+Na]+1; [M+NH4]+1; [M-H]-1; [M-H-H2O]-1
4. Group Compounds
   1. Mass Tolerance = 10 ppm
   2. RT Tolerance [min] = 0.2
5. Fill Gaps
   1. Mass Tolerance = 10 ppm

**Determining significance of metabolite quantitations with glucose tolerance and sampling**

Metabolite values as response variable were fit against each Nile rat in a mixed-effects linear model with the following models (written in R formula notation):

1. Metabolite quantitation ~ glucose tolerance \* sampling + (1|animal number)
2. Metabolite quantitation ~ sampling + (1|animal number)
3. Metabolite quantitation ~ glucose tolerance + (1|animal number)
4. Metabolite quantitation ~ glucose tolerance + sampling + (1|animal number)

Models 2, 3, and 4 were compared against model 1 to assess effect of each absent term in the formula. Model 2 assess effect of glucose tolerance. Model 3 assesses effect of sampling. Model 4 assesses the interaction between glucose tolerance and sampling. P-values were calculated using log-likelihood ratio test. P-values were adjusted using FDR correction (alpha = 0.05). Models were fitted, significance tested, and FDR-corrected using python package Statsmodels.

**Tracefinder targeted metabolomics method**

For data analysis, selected m/z and retention times were used to quantify metabolites (see Supplemental table), these peak areas were quantified using Thermo’s Tracefinder 4.0 application.

|  |  |  |  |
| --- | --- | --- | --- |
| Tracefinder ID | m/z | RT [min] | Adduct |
| Glutamine | 147.07649 | 12.646 | [M+NH4]+1 |
| Acetyl-L-carnitine | 204.12313 | 8.718 | [M+H]+1 |
| Leucine/Isoleucine | 130.08623 | 9.018 | [M-H]-1 |
| Leucine/Isoleucine | 130.08623 | 9.295 | [M-H]-1 |
| Proline | 116.07057 | 10.182 | [M-H]-1 |
| Carnitine | 162.11253 | 10.537 | [M+H]+1 |
| Threonine | 118.04984 | 12.041 | [M-H]-1 |
| L-Alanine | 88.0393 | 11.941 | [M-H]-1 |
| L-Phenylalanine | 164.07068 | 8.988 | [M-H]-1 |
| L-Arginine | 175.11904 | 18.052 | [M+H]+1 |
| Proline | 116.07092 | 10.143 | [M+H]+1 |
| Lysine | 147.11289 | 17.689 | [M+NH4]+1 |
| L-Serine | 104.0342 | 13.093 | [M-H]-1 |
| L-Tyrosine | 180.06563 | 11.137 | [M-H]-1 |
| Indole-3-acrylic acid | 188.07065 | 9.866 | [M+H]+1 |
| Nicotinamide | 123.05551 | 2.784 | [M+H]+1 |
| Propionylcarnitine | 218.13868 | 7.805 | [M+H]+1 |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Tracefinder compound table. | | | | | | |
| **Compound Name** | **Formula** | **MS Order** | **Precursor m/z** | **Peak Polarity** | **Adduct** | **Retention Time** |
| Nicotinamide | C6 H6 N2 O | ms1 | 123.0553 | Positive | M+H | 4.43 |
| O-Isovaleryl-L-carnitine | C12 H23 N O4 | ms1 | 246.17 | Positive | M+H | 8.55 |
| O-Butyryl-L-carnitine | C11 H21 N O4 | ms1 | 232.1543 | Positive | M+H | 9.06 |
| Propionylcarnitine | C10 H19 N O4 | ms1 | 218.1387 | Positive | M+H | 9.65 |
| Tryptophan | C11 H12 N2 O2 | ms1 | 205.0964 | Positive | M+H | 9.9 |
| Acetyl-L-carnitine | C9 H17 N O4 | ms1 | 204.123 | Positive | M+H | 10.43 |
| L-Phenylalanine | C9 H11 N O2 | ms1 | 164.0717 | Negative | M-H | 10.74 |
| DL-Leucine/Isoleucine | C6 H13 N O2 | ms1 | 130.0874 | Negative | M-H | 10.82 |
| Pantothenic acid | C9 H17 N O5 | ms1 | 218.1034 | Negative | M-H | 11 |
| Indole-3-acrylic acid | C11 H9 N O2 | ms1 | 188.0706 | Positive | M+H | 11.6 |
| DL-Proline | C5 H9 N O2 | ms1 | 116.0706 | Positive | M+H | 11.79 |
| L-Valine | C5 H11 N O2 | ms1 | 116.0717 | Negative | M-H | 11.85 |
| DL-Carnitine | C7 H15 N O3 | ms1 | 162.1125 | Positive | M+H | 12.05 |
| Guanosine | C10 H13 N5 O5 | ms1 | 282.0844 | Negative | M-H | 12.06 |
| L-Iditol to Six-carbon sugar alcohol | C6 H14 O6 | ms1 | 181.0718 | Negative | M-H | 12.48 |
| L-Tyrosine | C9 H11 N O3 | ms1 | 180.0666 | Negative | M-H | 12.76 |
| Glycine | C2 H5 N O2 | ms1 | 0 | Positive | M+H | 12.8 |
| L-Alanine | C3 H7 N O2 | ms1 | 88.0404 | Negative | M-H | 13.54 |
| Threonine | C4 H9 N O3 | ms1 | 118.051 | Negative | M-H | 13.6 |
| DL-Glutamine | C5 H10 N2 O3 | ms1 | 147.0764 | Positive | M+H | 14.11 |
| α-Lactose | C12 H22 O11 | ms1 | 360.15 | Positive | M+NH4 | 14.32 |
| Adenosine 5'-monophosphate | C10 H14 N5 O7 P | ms1 | 348.0704 | Positive | M+H | 14.39 |
| L-(+)-Citrulline | C6 H13 N3 O3 | ms1 | 176.103 | Positive | M+H | 14.49 |
| L-Serine | C3 H7 N O3 | ms1 | 104.0353 | Negative | M-H | 14.56 |
| Cytidine 5'-diphosphocholine | C14 H26 N4 O11 P2 | ms1 | 489.1146 | Positive | M+H | 14.88 |
| L-Glutamic acid | C5 H9 N O4 | ms1 | 146.0459 | Negative | M-H | 14.91 |
| L-Aspartic acid | C4 H7 N O4 | ms1 | 132.0302 | Negative | M-H | 15.14 |
| L(+)-Ornithine | C5 H12 N2 O2 | ms1 | 133.0972 | Positive | M+H | 19.29 |
| DL-Lysine | C6 H14 N2 O2 | ms1 | 147.1128 | Positive | M+H | 19.96 |
| L-(+)-Arginine | C6 H14 N4 O2 | ms1 | 175.119 | Positive | M+H | 20.67 |