NATIONAL CENTER FOR
QUANTITATIVE BIOLOGY
OF COMPLEX SYSTEMS

Nile Rat Plasma Metabolomics – fasting vs. random

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Project Lead:
Ben Anderson

Project Contributors:
Joshua J. Coon
Katherine A. Overmyer
Annie Jen

Collaborators:
Huishi Toh
Peng Jiang
James Thomson

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Objective

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_metabolites_table.xlsx contains the combined lipidomics and metabolomics data with p-values for each metabolite under significance testing.

Explanation of columns:

Unique ID: unique identifier for each metabolite
Type: metabolite or lipid
i: short unique ID
Proposed ID: Best estimate of metabolite ID.
Found in Tracefinder: TRUE if ID was found using Tracefinder.
Compound Class: Gives lipid or metabolite class for identified features, 'unknown' otherwise.
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 ID. Only used for metabolites.
Adduct: Adduct of m/z feature.
Polarity: Detected in positive or negative mode.
p-value columns (6 total columns):
First 3 columns are original p-values. 3 columns are false-discovery rate (FDR) corrected. Recommend using FDR corrected p-values. Blanks in p-value columns correlate with statistical models that fail to converge. See description of these columns in Data Analysis section (below).
Data Columns (60 total columns), e.g. 1076_10_FBG: sample formatted as [Animal#]_[week]_[FBG/RBG]. All values are log2-transformed.

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
    a. Upper RT limit = 22
2. Align Retention Times (ChromAlign)
    a. Reference file = 20210831_KAO_HILIC_T1082M_20210401_9wk_RBG
3. Detect Compounds
    a. Mass Tolerance = 10 ppm
    b. Min. Peak Intensity = 50,000
    c. Ions = [M+FA-H]-1; [M+H]+1; [M+Na]+1; [M+NH4]+1; [M-H]-1; [M-H-H2O]-1
4. Group Compounds
    a. Mass Tolerance = 10 ppm
    b. RT Tolerance [min] = 0.2
5. Fill Gaps
    a. Mass Tolerance = 10 ppm

Determining significance of metabolite quantitations with glucose tolerance and sampling

Linear mixed-effects models were fit using metabolite values as response variable, and glucose tolerance and sampling type as variables in the models. Data were fit using the following models (given in R formula notation; cf. page 44 https://cran.r-project.org/web/packages/lme4/vignettes/lmer.pdf):

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 vs. Model 1 assesses the effect of glucose tolerance. Model 3 vs. Model 1 assesses the effect of sampling (fasting vs. random). Model 4 vs. Model 1 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

Select molecules with known m/z and retention times were quantified as given in the table below. Peak areas were calculated 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