### IDENTIFICATION OF NOVEL BIOMARKERS OF GENETIC STATE IN MICE BY LC/MS AND METABONOMICS

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### Introduction

A metabonomics analysis approach is a powerful tool for discerning biomarkers in biological fluids such as urine and plasma. Such biomarkers have typically included those of a disease state or toxicological insult, and in the past, these group differences have been elucidated by sample analysis via 'H-NMR and GC/MS. Recently, an LC/MS alternative to the aforementioned techniques has been developed and is readily accomplished by using an orthogonal TOF MS and implementing an applications manager to allow the seamless acquisition and analysis of data. In this study, urine, collected in the morning and evening from white, black, and nude, male and female mice, was analyzed by LC/MS with subsequent data analysis by principal component analysis (PCA) and MS/MS for the elucidation of novel markers of sex and genetic state as well as the assessment of diurnal variations.

#### **Methods**

Urine samples were collected from black (C57BL19J), white (Alpk:ApfCD), and nude male and female mice (n=10) at two time periods, morning and evening for a total of 120 samples. A 100 µL aliquot of deionized water was added to each sample of mouse urine and was vortex mixed. All samples were then centrifuged at 13,000 rpm for 5 minutes at 10 °C and the supernatant liquid removed. A 50 µL aliquot of the supernatant was diluted with 150  $\mu\text{L}$  of distilled water and vortex mixed; the resulting solutions were transferred to an autosampler vial for analysis. The chromatography was performed on a Waters Alliance<sup>®</sup> HT equipped with a column oven and a Waters 2996 PDA detector. The HPLC system was coupled to a Waters Micromass<sup>®</sup> Q-Tof micro<sup>™</sup> equipped with an electrospray source operating in either positive or negative ion mode and a LockSpray<sup>™</sup> interface for accurate mass measurements.

### LC Conditions:

Lock Mass

Column	(2.1 x 100)mm 3.5 μm
Flow Rate	Symmetry C <sub>18</sub> 600 μL/min split to 150
	μL/min into MS
Injection Volume	20 μL
Gradient	Linear, 0-20%B over 0.5-
	4 min, 20-95%B over
	4-8 min, hold at 95%B
	for 1 min then return to
	0% B at 9 min (A =
	0.1% Formic Acid in
	Water: $B = 0.1\%$ Formic
	Acid in Acetonitrile)
	Gradient time increased
	to 45 minutes for MS/MS
MS Conditions	
M3 Conditions:	
Cone Voltage	40 V
Desolvation Temp.	150 °C
Source Temp.	100 °C
Collision Energy	10 eV
Detection Mode	Full Scan (100-1400 m/z)
Dwell	0.1 s
Collision Gas	Argon

Argon 50 fmol/μL Leucine Enkephalin in 50:50 Water: Acetonitrile (0.1% Formic Acid) at 200 μL/min

The resulting collected LC/MS data was analyzed using the MarkerLynx<sup>™</sup> Application Manager for MassLynx<sup>™</sup> 4.0 to discern ions of interest for further analysis by MS/MS with exact mass. The raw data was integrated and the detected peaks from each sample were used to construct a comprehensive list of all components in the analyzed samples. The processed data list was then analyzed by Principal Component Analysis (PCA) within the MarkerLynx program.

#### **Results and Discussion**

A representative total ion chromatogram for urine is shown in Figure 1 for both (a) positive and (b) negative ionization modes.



Figure 1. Total lon chromatograms for (a) positive and (b) negative ionization modes for urine from a male black mouse in the morning. Plots have been enlarged to show fine structure.

After data collection, the raw LC/MS spectra were processed using Principal Components Analysis (PCA). Figure 2 shows the resulting (a) Scores and (b) Loadings plots for urine collected in the morning and evening from female white mice. The Scores plot in Figure 2a clearly shows the clustering of the samples into two groups, urine collected in the morning and urine collected in the evening. Figure 2b displays the corresponding Loadings plot for the PCA, which facilitates the elucidation of target ions (circled in red) for future MS/MS experiments.



Figure 2. Scores and Loadings plot for urine collected in the morning and evening from female white mice. The Scores plot (a) shows clustering of morning and evening samples, whilst the Loadings plot (b) aids in the selection of marker ions for future MS/MS experiments.

In addition to observing group clustering due to diurnal variations (Figure 2), similar PCA results can be obtained which reflect gender differences (Figure 3) amongst black mice and variation in genetic state (Figure 4).



Figure 3. Scores plot showing clustering into male and female groups based on gender.



Figure 4. Scores plot illustrating clustering based on genetic state.

In addition to obtaining group cluster information from a Scores plot, the Loadings plot provides a rapid means to identify ions responsible for group clustering, thus allowing selection of ions for further investigations via MS/MS and subsequent biomarker identification. Figure 5a-c shows extracted ion chromatograms for the m/z = 131 ion for (a) black, (b) white and (c) nude male mice as chosen from the corresponding Loadings plot.



Figure 5. Loadings plot showing ions responsible for clustering amongst black, white and nude male mice. Expanded area shows extracted ion chromatograms for m/z = 131 ion for (a) black, (b) white and (c) nude mice.

As illustrated in Figure 5, the m/z = 131 ion is present in black and white mice, but not in nude mice, and could be a potential biomarker to identify genetic differences in mice.



Figure 6. Elemental composition calculation results for the m/z = 131 ion. The most reasonable molecular formula is  $C_6H_{11}O_3$ .

The m/z = 131 ion was selected for further MS/MS experiments, and displays the fragmentation pattern shown in Figure 7.



Figure 7. Resulting spectrum from MS/MS of m/z = 131 ion for a urine sample from a white, male mouse. The collision energy was 20 eV.

Using the fragment information obtained from the MS/MS experiment, exact mass data and elemental composition, the endogenous metabolite is proposed to be one of the 5 compounds shown in Figure 8 having the elemental composition  $C_6H_{11}O_3$ . Confirmation of metabolite identity using LC/MS of the standard compounds is currently underway.



Figure 8. Proposed structures for the m/z = 131ion based on the elemental composition  $C_6H_{11}O_3$ . (a) DL-mevalonic lactone, (b) 3-methyl-2-oxovalerate, (c) pantolactone, (d) 2-oxoisocaproate, (e) 2-acetolactate.

### Conclusions

- Rapid gradient LC/MS and multivariate analysis has been employed to identify diurnal, gender and strain differences amongst black, white and nude mice.
- Targeting potential endogenous metabolites for future structural confirmation is facilitated by Principal Component Analysis (PCA) of LC/MS data.
- Confirmation of metabolite structure using LC/MS/MS experiments and authentic standards are currently in progress to identify the m/z = 131 ion as well as other biomarkers.

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