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Jessica Maya

J Daniel Sharer

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Jessica Maya[1] and J. Daniel Sharer[2]

[1]Department of Biology, University of Alabama at Birmingham, Birmingham, AL, USA
[2]Department of Human Genetics, Emory University School of Medicine, Atlanta, GA, USA

Abstract
The purpose of this experiment was to create personalized metabolomic profiles for types of obesity based on organic acid levels. Three groups of mutant mice (*ob/ob*, *Mc3r* knockout, and *Mc4r* knockout) and a control group known as studs were used to create the profiles. Multiple urine samples were collected and compared between the groups. Initial preparations were made for the testing of organic acids, which were the metabolites used for the profiles, and the samples were processed through a gas chromatograph-mass spectrometer. The resulting levels of 33 organic acids, including two internal standards and control levels, were analyzed. The data were interpreted and the concentrations of acids were graphed. Potentially, after finding the averages of each group, the profiles could be used to correctly diagnose other mice with matching metabolomic profiles depicting a type of obesity. This would represent a less intrusive and more personal type of medical tool that could be used in everyday diagnosis of metabolic diseases.

Keywords: Stud, *ob/ob*, *Mc3r*, *Mc4r*, gas chromatography, mass spectrometry

Introduction
More than 60 % of adults in the United States are classified as overweight or obese. While the most common cause of obesity is a dietary surfeit of calories, there are some types of obesity that are a result of treatable pathologies.[2] Because of this fact, accurate and discerning diagnostics are very important when trying to treat a patient's obesity successfully. This study focuses on the use of metabolomic profiling to diagnose types of obesity in a set of mice. In this method, a blood or urine sample is taken from a patient and tests are performed in order to determine characteristics of the individual's metabolism and thereby to inform a diagnosis.[1] Because it is noninvasive, focused on changes in systems that are affected by many different disease pathways, and quantitative, this method of profiling could become a useful type of diagnostic test for a number of different diseases.

This study's focus was the metabolomic profiling of different groups of obese mice according to their levels of organic acids. The groups representative of obesity were *ob/ob* mice, melanocortin 3 receptor (*Mc3r*) gene knockout mice, and melanocortin 4 receptor (*Mc4r*) gene knockout mice. As a preliminary step, however, summary investigations were made of the current use of metabolomic profiling in medicine and the current state of knowledge regarding the human analogues of the three mouse obesity groups.

Metabolomic Profiles Used in Modern-day Medicine
The uses of metabolomic profiling have been demonstrated in many recent studies. Metabolomics is basically an "approach that attempts to profile all the metabolites in a biological matrix." [2] It is analytical in nature and has been applied mostly to animal studies thus far, with only a few human studies to date. Mass spectrometry is the most widely used tool for this type of analysis, though gas and liquid chromatography are also common. Medical disorders for which profiling is thought to have particular promise include diabetes, obesity, and other associated disorders. In one study, the profiles of leptin-deficient mutant mice were recorded and analyzed.[3] The study was able to relate increased levels of leptin to energy homeostasis, glucose homeostasis, and overall obesity. It concluded that leptin was able to be analyzed using this method and that obesity was associated with its deficiency.

The Role of ob/ob
Leptin is a hormone that is secreted by adipose tissue to signal the neuroendocrine system that body energy stores are low. It is released in proportion to fat mass and regulates appetite as well as the activity of the thyroid, adrenal, pituitary, and other related glands. As a result, leptin deficiency causes over-eating and sluggish physical activity.[4] The discovery of this hormone in the 1990s gave hope to the medical community that a portion of people with obesity could be helped with the manipulation of this hormone. Individuals with an *ob/ob* genotype are homozygous in a certain allele that causes non-functionality of leptin receptors. It is a gene mutation known as the "obesity gene" that is "responsible for a severe syndrome of obesity with insulin resistance."[5] The gene encodes a fat-specified mRNA and a protein, which is regulated in adipose tissue. Thus, leptin can be present in the *ob/ob* individual, but the receptor/binding sites do not work such that leptin signaling is affected. Early diagnosis of a leptin deficiency is important, as this type of condition is treatable in a different way than primary obesity. A supplement of leptin along with controlled diet and exercise can lead to a quicker and more successful treatment of the patient's condition.[14]

The Role of Mc3r

The melanocortin 3 receptor gene has been found to impact the regulation of weight gain and can be a predisposing factor to obesity if mutated. The pathway of melanocortin regulates energy by stopping over-eating, increasing energy expenditure, and reducing the storage of energy.[6] It was found that mice deficient in the *Mc3r* gene had increased body fat and decreased lean mass, which was "not caused by increased food intake but arose from increased feed efficiency."[7] Metabolic rates in these mice were found to be the same as normal rates; hormones released from the thyroid were undisturbed, and the respiratory exchange rate was unchanged as well. The causes of their obesity were therefore their susceptibility to high fat diet-induced obesity and their inactivity. Another study also confirmed the *Mc3r* knockout mutation as an associative gene to obesity. Research also shows that *Mc3r* can be a predisposing gene that contributes to an increased adiposity, or fat cell accumulation, which could indicate pre-obesity in a subject.

The Role of Mc4r

Mutations in the melanocortin 4 receptor gene are the most frequent monogenic causes of severe early onset obesity. In humans the *Mc4r* mutation results in a syndrome of hyperphagic obesity that can be present with either dominant or recessive inheritance patterns. There is also a marked increase in bone mineral density, which affected all subjects in one study.[8] Although research is still being done on the prominence of the mutation of the *Mc4r* gene in severe obesity cases, there are studies that suggest that this gene is more significant than previously thought since it can be influential in both a dominant and recessive state. As of now, genetic testing is the only method for definitive diagnosis of *Mc4r*-linked obesity. It is important to diagnose this type of obesity early because, without aggressive treatment, it is very unlikely to be resolved. The knowledge of this condition allows patients to fully realize the importance of their everyday eating behavior.

This Study's Hypothesis

The working hypothesis of this study is that the type of obesity in a random sample can be correctly diagnosed solely by metabolomic profiling of organic acids.

Materials and Methods

Materials

A high performance liquid chromatograph (HPLC) with a tandem mass spectrometer (Micromass Quattro micro API Technologies) was used to test creatinine levels, along with proprietary software (Masslynx). Quality control samples (high and low) were also provided by the UAB Genetics Lab to make sure the instrument was in working condition and was reading the levels correctly for both the high and low ranges of levels. The animals used were C57BL/6 mice as the stud group of mice, *ob/ob* mice, *Mc3r* knockout mice, and *Mc4r* knockout mice. In order to read the organic acid levels, an organic acid extraction kit was used along with the BGL Library for organic acid level readings and the software linked to the chromatograph.

Animals

The size of each group of mice varied from 3 to 6 useable profiles: 6 studs, 4 *ob/ob*, 5 *Mc3r* knockout, and 3 *Mc4r* knockout.

Creatinine Determination

Urine samples were gathered from the obese mouse lab. These included urine samples from stud mice, *ob/ob* mice, *Mc3r* knockout mice, and *Mc4r* knockout mice. All were placed on ice to be tested in the genetic metabolomics lab. The creatinine levels, measured in micromoles, were determined by gathering 2 µL to 5 µL of the urine sample and running it through an HPLC. The observed levels were used to determine the volume of urine to use in the actual testing of organic acids, i.e., the volume containing 1 µmol of creatinine.

Extraction

These amounts were aliquoted into extraction tubes, and quality controls were added that acted to keep the organic acid levels in a testable range. 0.4 mL of 5 M NaOH and 0.8 mL of hydroxylamine were added. The volume of each sample was raised to 1.5 mL by adding deionized water, and each extraction tube was vortexed. The tubes were capped and heated in a water bath at 70 °C for 30 min. The tubes were then cooled for 10 min at room temperature. Then, 0.8 mL of 5 M HCl was added, and 0.2 mL of 2.5 mM α-ketocaproic acid and 0.2 mL of 1 mM tetracosane were added to each tube as internal standards. The samples were extracted with 6 mL of ethylacetate:ether. The tubes were then placed into the Vortex mixer for 10 min. The extraction tubes were centrifuged for 1 min. The lower aqueous layer of the samples was then removed and discarded. Two mg of sodium sulfate was added to the extract. The tubes were mixed thoroughly and centrifuged for 3 min. The anhydrous extracts were transferred into derivatization tubes and placed on a heating block at 50 °C to 55 °C. Each tube was dried under a stream of nitrogen for 25 min.

Derivatization

Approximately 0.2 mL of the derivatization mixture was added to the dry extract in order to enable chromatographic separation. The tubes were capped tightly, vortexed, and heated in a heating block at 80 °C to 85 °C for 30 min. The tubes were cooled to room temperature and transferred to individually labeled autosampler vials. The vials were taken to the gas chromatographer for testing.

Analyzing the Data

Once the quality controls were checked, the sample data files analyzed using the software packaged with the

chromatograph. The spectrum for the integrated peaks was analyzed. The integration for each individual peak, signifying a level of a particular organic acid in moles, was checked and corrected manually if necessary. The resulting levels of each organic acid present were transferred into graphical analysis software to be better investigated and compared. The 33 organic acids analyzed were as followed: Lactate, glycolic acid, glyoxylic acid, oxalic acid, pyruvic acid, 3-hydroxybutyric acid, 2-hydroxy isovaleric acid, ketoisovaleric acid, acetoacetic acid-diTMS (peak 1), acetoacetic acid (peak 2), acetoacetic acid-diTMS (peak 3) 2-keto-3-methylvaleric acid, ethylmalonic acid, ketoisocaproic acid, succinic acid, 2-ketocaproic acid diTMS, glyceric acid, fumeric acid, glutamic acid, 3-methylglutamic acid, adipic acid, hydroxyproline, 2-hydroxyglutarate, 3-hydroxy-3-Methylglutaryl-CoA, 2-ketoglutaric acid, hydroxyPAC, SUB, aconitic acid, citric acid, sebacic, phydroxylactic acid, phydroxypyruvic acid, and the control compound tetracosane (c-24; internal standard).

Data Collection and Analysis

Once all data were acquired for each type of obese mouse as well as the stud mice, the levels for each acid were averaged within the groups. Metabolomic profiles were made with these means. Standard deviations for the levels of each acid were also computed to facilitate comparison among individuals. These profiles were then used to generate conclusions regarding the type of obesity represented in previously gathered samples of urine. Thus, it was possible to test the reliability and specificity of metabolomic profiling as a diagnostic method.

Results

Organic acid metabolomic profiles were generated to show the normal (95 % population-inclusive) range of the levels of each organic acid for each mouse type. The averages and standard deviations of each organic acid were found. Then, a range was calculated by adding and subtracting two standard deviations from the mean.

Figures 1-4 display the graphs of the ranges of normal organic acid levels in each type of mouse. These are the working profiles for the mice. The lighter bars indicate the upper bound, and the darker bars indicate the lower bound.

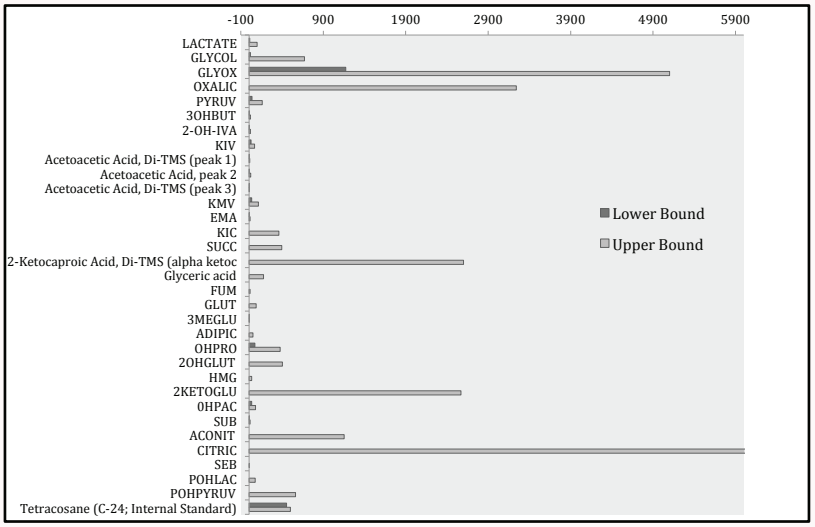

Figure 1. Stud Profile. These are the 33 organic acid levels that make up the profile of the stud group of mice at a 95 % accuracy range.

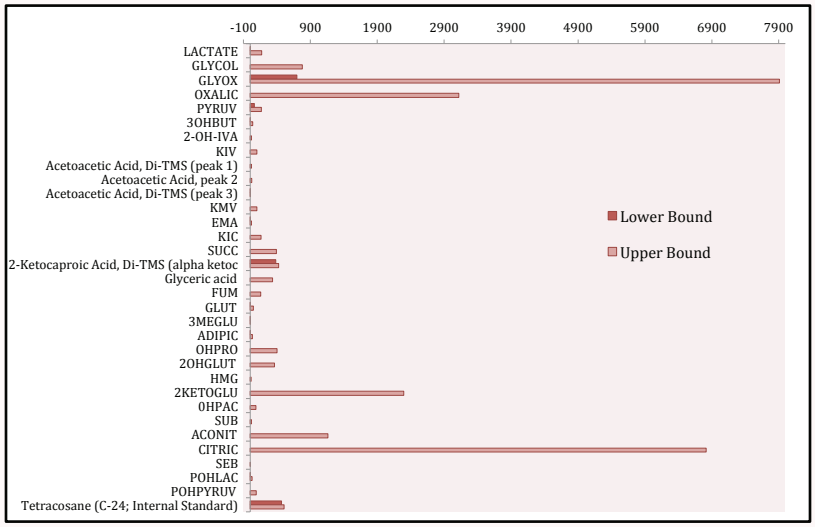

Figure 2. ob/ob Profile. These are the 33 organic acid levels that make up the profile of the ob/ob group, or leptin deficient group, of mice at a 95 % accuracy range.

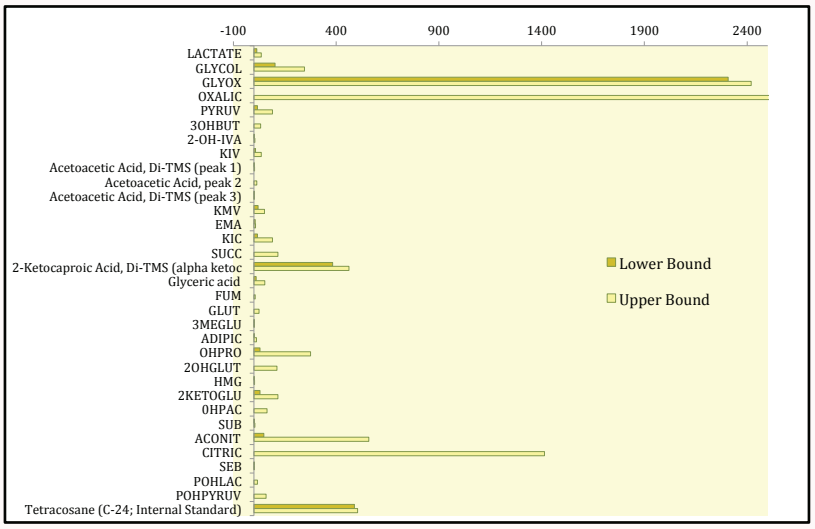

Figure 3. Mc3r *Profile. These are the 33 organic acid levels that make up the profile of the* Mc3r *group of mice at a 95 % accuracy range.*

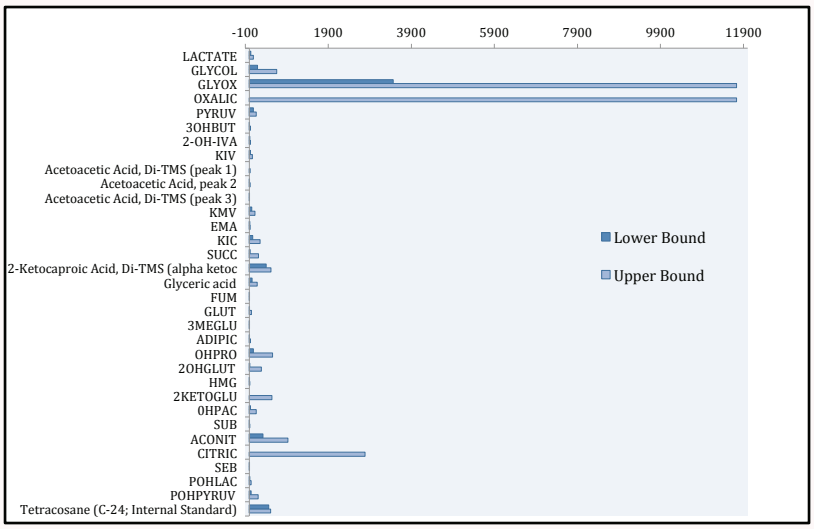

Figure 4. Mc4r *Profile. These are the 33 organic acid levels that make up the profile of the* Mc4r *group of mice at a 95 % accuracy range.*

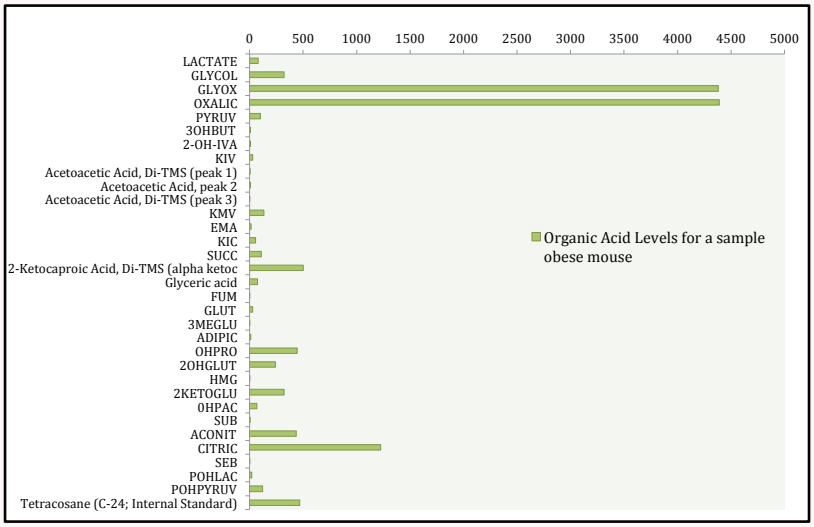

Figure 5. Organic Acid Levels of Unknown Obese Mouse Sample. This figure shows the 33 organic acid levels of an unknown sample of an obese mouse that will be compared to the three profiles created in this project.

Next, a metabolomic profile was generated from a randomly-selected urine sample from one of the three mutant mouse types.

The random sample profile was compared to the three population standard profiles to allow its identification with (i.e., a diagnosis of) one of the three types of obesity under study.

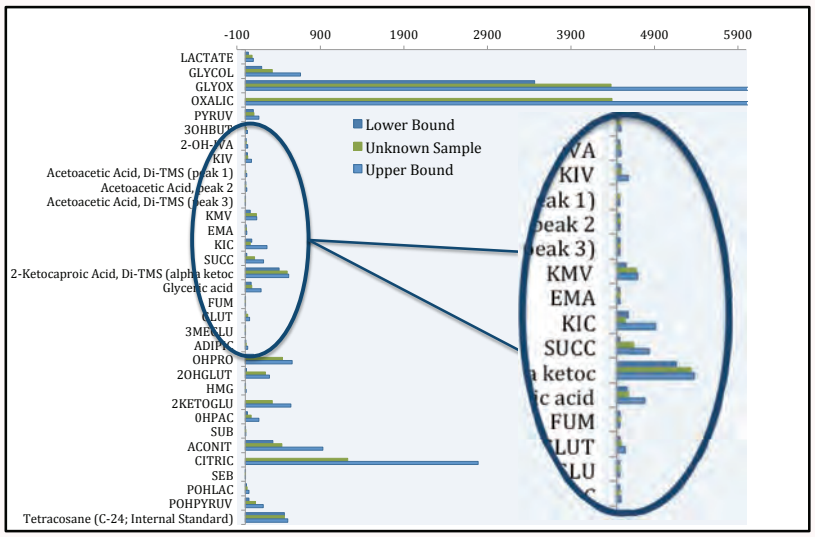

Figure 6. Mc4r *Profile Compared to Unknown Sample. When compared to the unknown sample,* Mc4r *fit the sample nearly perfectly, having only one or two discrepancies that went above or below the 95 % boundaries.*

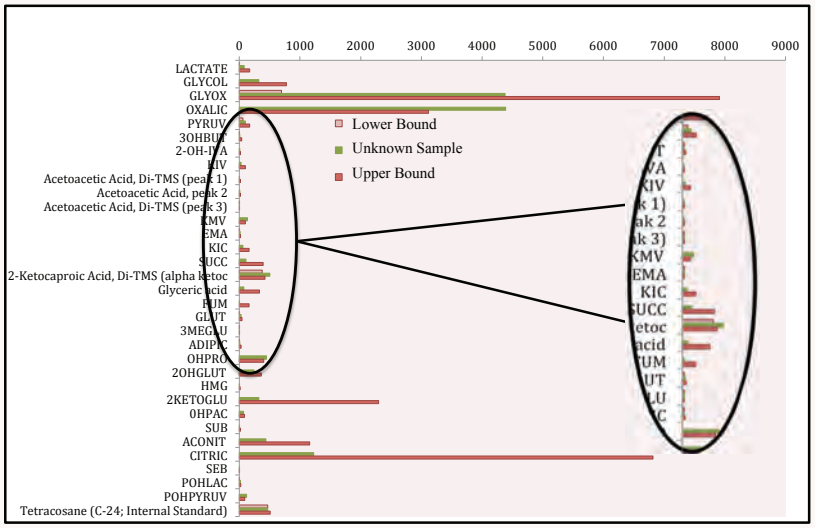

Figure 7. ob/ob *Profile Compared to Unknown Sample. When compared to the* ob/ob *profile, it can be seen that in more than 5 organic acid levels were higher than what the upper bound dictates. These include oxalic, kmv, OHPro, hmg, and POH Pyruvic acid.*

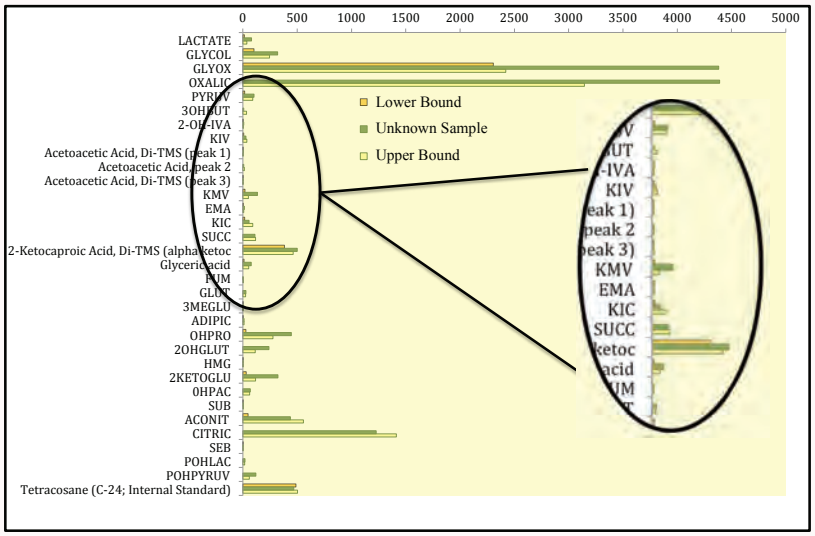

Figure 8. Mc3r *Profile Compared to Unknown Sample. In the* Mc3r *profile, multiple levels were much higher than the profile illustrates, and so this type of obesity can automatically be removed as a possible diagnosis of this sample.*

Comparison of the random sample profile revealed a highly specific and strong pattern of similarity with the standard *Mc4r* profile (Figure 4), which is seen particularly easily, for example, by examining the levels of glyoxylate acid and oxalic acid.

Discussion

Because a strong and specific pattern of similarity was observed between the random sample profile and the standard *Mc4r* profile (i.e., the levels of organic acids in Figure 5 could only fit the profile of the *Mc4r* mouse in Figure 4, and not that of any other mouse type), the hypothesis was

confirmed and reliable, selective diagnosis was possible. Thus, diagnostically useful metabolomic profiles can be created for genetic variants of obesity using organic acids as the only metabolites profiled, such that the type of obesity in a random sample can be correctly diagnosed solely by the profiles produced. The results of this project suggest that metabolomic profiling is a possibility for clinically useful obesity diagnoses given multiple samples and adequate control of potential confounding variables.

One variable other than the obesity gene that could affect the profiles is the time of day at which each urine sample was collected: the mouse variants may have different eating habits, and the levels of certain organic acids could fluctuate as a function of the time elapsed since a meal. The age of the mice is another variable, but since most of these mice samples were collected at a young age before the onset of obesity, this is not a particular problem for this study.

Further study in this area is needed to understand the effects of the diets, collection times, and habitat conditions of the mice. Increasing the sample sizes for each mouse population would give the data more precision. Other diseases could also be studied to see if the same diagnostic method used here could also be used to diagnose other illnesses. Finally, other metabolites such as amino acids could be tested as metabolomic profile analytes to determine whether they allow for more accurate diagnosis.

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Addendum: Definitions of Terms and Abbreviations

Studs: Term used in the lab for the control mice. These are C57 black 6 mice to be compared to the mutant mice.

Leptin: A protein hormone that regulates energy intake and expenditure.

ob/ob: These mice are leptin deficient, causing their appetites to increase and their metabolisms to decrease. This leads to obesity.

Mc3r (Oregon homozygous): This mouse type has a knockout of the melanocortin 3 receptor (*Mc3r*) gene in the brain. When this gene is inactivated, it causes increased fat mass and reduced lean mass, so the mice are longer but not as seemingly fat as *ob/ob* mice. They are still considered obese.

Mc4r (Southbeach mutation): The mutation of the melanocortin 4 receptor (*Mc4r*) seen in these mice causes a change in the control of appetite and general eating behavior. It causes over-eating and is the common case of human obesity. It is also inheritable obesity.

Gas Chromatography Mass Spectrometer: A machine used to separate and identify different components of a sample, in this case, organic acids. The chromatograph separates the different components of the sample, then the spectrometer ionizes the components in a sample by a direct bombardment of electrons, and software is used to detect and quantify levels of each organic acid in the sample.