Supplemental Methods

LC/MS/MS Analysis of Acetaminophen (APAP) Metabolites

Hepatic tissues were placed in twice the volume of normal saline per tissue weight (in grams), and were homogenized by sonication. Control mouse plasma was used to dilute urine or bile 1000-fold, and serum or hepatic homogenate 100-fold before analysis. The protein in each 50 µL sample was precipitated by addition of 50 µL of 50:50 methanol:water plus 150 µL of 10 µg/mL acetylsalicylic acid, which served as an internal control, in 50:50 methanol:acetonitrile. The mixture was vortexed for 5 minutes, centrifuged at 2500xg for 20 minutes, and 200 µL supernatant was obtained for LC/MS/MS analysis. 15 µL of each supernatant was injected into a Thermo Hypersil BDS C-18, 5 µm 50x2.1 mm column with Thermo Hypersil BDS C-18 Javelin guard column attached to an Applied Biosystems Sciex 4000 LC/MS system. Metabolites were ionized either in the positive or negative modes. The daughter ions were monitored and separated though a mixed step and linear gradient at a constant flow rate of 0.4 mL/min. Mobile phase A consists of 5 mM ammonium acetate with 0.1% formic acid. Solvent B consists of 100% methanol. The gradient is set at 0% B for 1.5 minutes, and increased to 28% B at 1.6 minutes until 3 minutes. The gradient was ramped up to 50% B at 4.1 minutes, where it remained until 5 minutes, when it was decreased to 0% B at 5.1 minutes and equilibrated to the end of run at 6.5 minutes. Metabolite concentrations were determined by comparing the integrated peak areas with those generated using reference standards obtained from the following sources: APAP, Hawkins, Minneapolis; APAP-Glucuronide (APAP-Glu), Sigma-Aldrich; APAP-Sulfate

(APAP-Sul), Sigma-Aldrich; APAP-Mercapturate, Richman Chemicals Inc., Lower Gwynedd, PA; APAP-GSH, Toronto Research Chemicals Inc., North York, Canada, and APAP-Cysteine (APAP-Cys) was synthesized at Roche. A liver tissue density of 1.05 g/mL was used in calculating hepatic metabolite concentrations(Sohlenius-Sternbeck 2006).

LC/MS/MS Analysis of Methionine and Glutathione

Snap-frozen hepatic tissues were homogenized in 800 uL solution (per 50 mg liver tissue) composed of formic acid (0.1% v/v), KCL (1.15% w/v), EDTA 1mM, Bathophenanthrolinedisulfonic acid disodium salt (BPDS) 2mM, and then centrifuged at 16,000xg at 4^{o}C (Bouligand et al. 2006). The supernatants were harvested and spiked with glutathione-13C215N and methionine-d3 (Cambridge Isotope Laboratories, Inc) as internal standards. The samples were analyzed by HPLC-MS/MS using multiple reaction monitoring (MRM) as described (http://www.asms.org). Briefly, samples were subjected to a zic-HILIC column 5 µm, 2.1 mm i.d. x 150 mm length (Sequant, Switzerland). A Shimadzu binary HPLC system delivered a gradient at flow rate 0.4 mL/min with mobile phase A (20mM ammonium acetate in H2O) and B (acetonitrile). The gradient was as follows: 20% A at 0 min; linearly increased to 40%A in 1.8 min; went up to 80% A at 2min; stayed at 80% A till 2.5 min; dropped to 20% A at 2.8 min; 20% A till 4min. Analyte detection was performed using a Ultima triple quadrupole mass spectrometer (Waters, USA). The analytes were detected in positive ion mode, with dwell time set between 0.25 and 0.5s, and the inter-channel delay was 5 ms. The ion transitions monitored by m/z were

308/178.9 for glutathione, 312/182.9 for glutathione-13C215N, 150/104 for methionine, and 153/107 for methionine-d3.

Metabolomic Profiling by NMR

Frozen liver tissues (~500 mg) were harvested and processed as described (Zheng et al. 2007). 2D NMR spectra were recorded, processed, and NMR assignments were initially made by comparison with reference compound data (Ulrich et al. 2008). The assignments were then confirmed by acquiring the spectra of samples spiked with reference compounds, as described previously (Zheng et al. 2007). The algorithm described in Zheng et al. (Zheng et al. 2007) was used to align the peaks occurring in different spectra that corresponded to the same metabolite. The aligned peak clusters were then filtered using the following criteria: i) the metabolite peak was absent in no more than 4 samples obtained from the different strains and time-points; ii) the maximal intensity of the peak was larger than 10; iii) the p-value after ANOVA testing was smaller than 0.001; iv) the peaks within a defined spectral region (13C: 61-81; 1H: 3.3-4.3) were excluded due to a high degree of peak overlap. The p-values after ANOVA testing of peaks indicated whether a difference in peak intensities among the strains at the indicated time points was significant. For each of the 12 conditions (4 strains at 3 time points) examined, and each had 3 or 4 biological replicates, the ANOVA test was used to determine whether the average metabolite intensities in these 12 conditions were significantly different. Finally, the peaks meeting these criteria were ranked based upon the fold change of the maximal intensity to

the minimal intensity. Over 400 metabolite peaks were quantified and analyzed by this method.

Gene Expression Analysis

Livers were snap frozen in liquid nitrogen before homogenization. The samples were processed according to our published methods (Klein et al. 2004). Briefly, total RNA was isolated from frozen liver tissues by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Trizol, Invitrogen) plus LiCl precipitation. Aliquots of the resulting RNAs were purified using RNeasy columns (Qiagen) and 10 μg of each purified total RNA served as template for reverse transcription using a T7-promoter coupled oligo(dT) primer, followed by an in vitro transcription reaction (Affymetrix, version 2.03). The labeled samples were applied to the Affymetrix Murine Genome MOE430v2 chip and processed according to the manufacturer's instructions. The resulting image files were analyzed using Affymetrix Microarray Analysis Suite version 5.0 software. Experiments were performed on tissue samples obtained from three individual mice for each strain analyzed at the indicated time points. The probe intensity data generated from all arrays were read into the R software environment (http://www.R-project.org) directly from the .CEL files using the R/affy package (Gautier et al. 2004), which was also used to extract and manipulate probe level data to assess data quality and to create expression summary measures. The array data were also checked for quality using GCOS (Gene Chip Operating Software) from Affymetrix. Normalization was carried out using the robust multiarray average (RMA) method (Irizarry et al. 2003) to generate one expression measure for each probe set on

each array. To measure the temporal difference after the administration of APAP, for each mouse strain, replicate data obtained from different time points were compared using the student t-test. The empirical Bayes method (Lönnstedt et al. 2002; Smyth 2004) was applied to adjust the standard deviation estimation of each probe on the chip and FDR-adjustment proposed by Benjamini and Hochberg (Benjamini et al. 2001) was applied to adjust the p-values for multiple testing. Gene expression changes meeting pre-determined criteria (fold-change > 2 and adjusted p-value <0.01) were selected for further analysis. This data is available in the NCBI GEO database under accession number: GSE17649.

Pdk4, *Bhmt2*, and *G6pc* mRNA expression were further analyzed using two-step SYBR Green real-time PCR as described by the manufacturer (Applied Biosystems, Foster City, CA) using the following primers. All expression measurements were normalized relative to that of β-Actin.

	Forward	Reverse
Pdk4	GATCAGGGCAGTGACTTTCACA	AGATTACCCAAATGCTGACAGTTTT
Bhmt2	CTGATGGCCAAGGACCGTAT	CATTGGTGATTAAGTTTGGCATAAA
G6pc	GCTGTGTCTGGTAGGCAACTGT	TTTTGCCCAAGATCTCAGAGTTT

Preparation of S-methyl Methionine (SMM) Chloride

Ten grams of L-SMM iodide (Sigma, St. Louis, MO) was dissolved in 100 mL water, and applied to a 5 cm diameter column packed with Dowex 1 (OH-). HCl was added to the

eluant until the pH was neutral, and then the L-SMM-Cl containing solution was lyophilized to dryness.

Preparation of [^{14}C]-L-methionine Methylsulfonium Iodide ([^{14}C]-SMM)

[^{14}C]-Methionine (Moravek Biochemicals, 2.52 mCi, 53 mCi / mmol, 0.046 mmol in 2.9 mL 7:3 EtOH-H2O solution, 90% L-enantiomer) was concentrated by rotary evaporation. The residual white solid film (9.0 mg) was re-dissolved in 300 µL water, and rinsed twice with 200 µL of water into a 4 cc 'V' vial. Methyl iodide (50 µL, 0.8 mmol) was added, and the clear colorless two phase reaction mixture was swept with nitrogen, sealed, and immersed in a 47 °C oil-bath. After stirring for 15 h, radio-TLC analysis of the single phase solution indicated that 95% of the activity was [^{14}C]-SMM (Rf 0.2) and 5% of the activity was at the Rf (near the solvent front) belonging to the starting material. An additional 20 µL of methyl iodide was added, and the reaction was continued at 47 °C for 4h. Radio-TLC indicated 98% product and 1.7% starting material. The 'V'-vial was fitted with a 14/20 F adaptor and the crude reaction mixture was concentrated by rotary evaporation. The dry colorless residual film was re-dissolved in 150 µL water. Ethanol (850 µL) was added (< 1 min) while stirring at ambient temperature. The clear colorless solution became cloudy after five minutes. After 15 more minutes of stirring at ambient temperature, the partially crystallized (fine white crystals) solution was refrigerated (0 °C) overnight. The cold mixture was compacted by centrifuge (2 min), and the clear, colorless supernatant was removed by pipette. The residual white solid was dried (< 1 mmHg, ambient temperature), and re-dissolved in 150 µL water. This solution was sterilized by rinsing with 4.85 mL of water

through a 0.45 μ filter into an ethanol rinsed and nitrogen dried septum vial. This solution was gently bubbled with nitrogen (2 min) to afford 1.28 mCi of [^{14}C]-SMM with specific activity of 50.9 mCi/mmol. The radiochemical, chemical and enantiomeric purity of [^{14}C]-SMM was 97.8%, 98.2%, 90.3%, respectively. TLC: Silica gel: 20% NH4OH, 80% MeOH. LCMS: [M+H]+ 268. Chiral purity by HPLC was performed on Chiral OD-RH 4.6x150mm, A: 0.1% TFA in H2O, B: 0.1%TFA in acetonitrile at 1 mL/min, 25% B isocratic, 30 0C, 220 nm (Fmoc derivative).

Supplemental Table and Figure Legends

Supplemental Figure 1. Principal component analysis (PCA) of metabolite profiles in mouse liver. Over 400 metabolite peaks were quantified and analyzed by ^{1}H-^{13}C 2-dimensional-NMR analyses in liver samples obtained from 4 inbred strains at 0, 3 and 6 hr after acetaminophen exposure. The C57BL/6J mice were extremely moribund at the 6 hr time point (Fig. 1B). We also observed that a large percentage of the metabolite peaks had a very low abundance level in these samples, which was consistent with their severely moribund state at this time. Therefore, the metabolic response of C57BL/6J mice at 6 hour, which was quite different from the other mice, was removed from the subsequent PCA analyses. The metabolite peaks were then filtered using criteria described in the supplemental Materials and Methods. PCA indicated that the top 50 metabolite peaks could distinguish the metabolomic response of the resistant SJL/J strain from the 3 sensitive strains 3 hour after APAP exposure. Data points that are more similar to each other are more proximately located on the graph; the distance between points is an indication of similarity. (*A*) The PCA results for the top 50 metabolites are plotted; the first principal component explained 48.8% of the total variance and the second one explained 27.5%. Each data point corresponds to the composite metabolomic data for an individual mouse at that indicated time point. A red, blue, or green box indicates a sample obtained at 0, 3, or 6 hrs, respectively. The squares, circles, triangles, and stars represent the C57BL/6J, DBA/2J, SJL/J, and SM/J strains, respectively. The metabolite profiles of the resistant

JL/J strain 3 hour after acetaminophen exposure clustered (along PC1) with the profiles of all of the strains prior to drug treatment. This indicates that the 3 hour time point differentiates the response of the resistant SJL/J strain from that of the 3 sensitive strains. (*B*) A loading plot is used to indicate the contribution of each metabolite peak to PC1 and PC2. Each square represents one of the 50 metabolite peaks analyzed; and the following metabolites are indicated by color: alanine in pink, betaine in red, GSH in yellow, and lactate in green. For instance, the red metabolite peak at the left (betaine) made the largest contribution for PC1 (~0.8 in magnitude) and also a moderate contribution for PC2 (~0.2 in magnitude).

Supplemental Figure 2. Changes in the mRNA levels of DNA damage response genes in liver after APAP treatment. An increase in *Ddit3*, *Dnajb1*, *Gadd45a* and *Gadd45b* mRNAs are indicators of DNA damage. The level of expression of these 4 mRNAs in liver prepared from 4 inbred strains was analyzed at 0, 3 and 6 hrs after treatment with a single 300 mg/kg dose of APAP. The level of expression of all 4 mRNAs was increased in resistant SJL/J mice, as well as in the other 3 strains that are sensitive to APAP-induced liver injury. Each bar represents the average ± standard deviation of three measurements prepared from independent samples.

Supplemental Table 1. Acetaminophen (APAP) and its metabolites were analyzed in serum, bile, urine and liver obtained from SJL/J or B10.D2 mice 1 or 2 hr after an i.p.

injection of 300 mg/kg APAP. At 1 hour, there were no differences in the biliary or urinary concentration of APAP or its metabolites between the two strains. However, SJL/J mice had a higher biliary APAP-GSH level than B10.D2 mice at 2 hour (4.98 ± 1.08 vs. 1.33 ± 0.11 mM), but there were compensatory increases in biliary APAP-CYS, APAP-GLU, and APAP-SUL levels in B10.D2 mice at the 2 hr time point. The values shown represent the mean ± standard error for n = 4 ~ 5 independent measurements. An underlined value indicates that at least one measured value was below the minimum required for quantification. N.D. indicates that the metabolite was not detected, and a * indicates a p-value < 0.05 for the differences between the two strains.

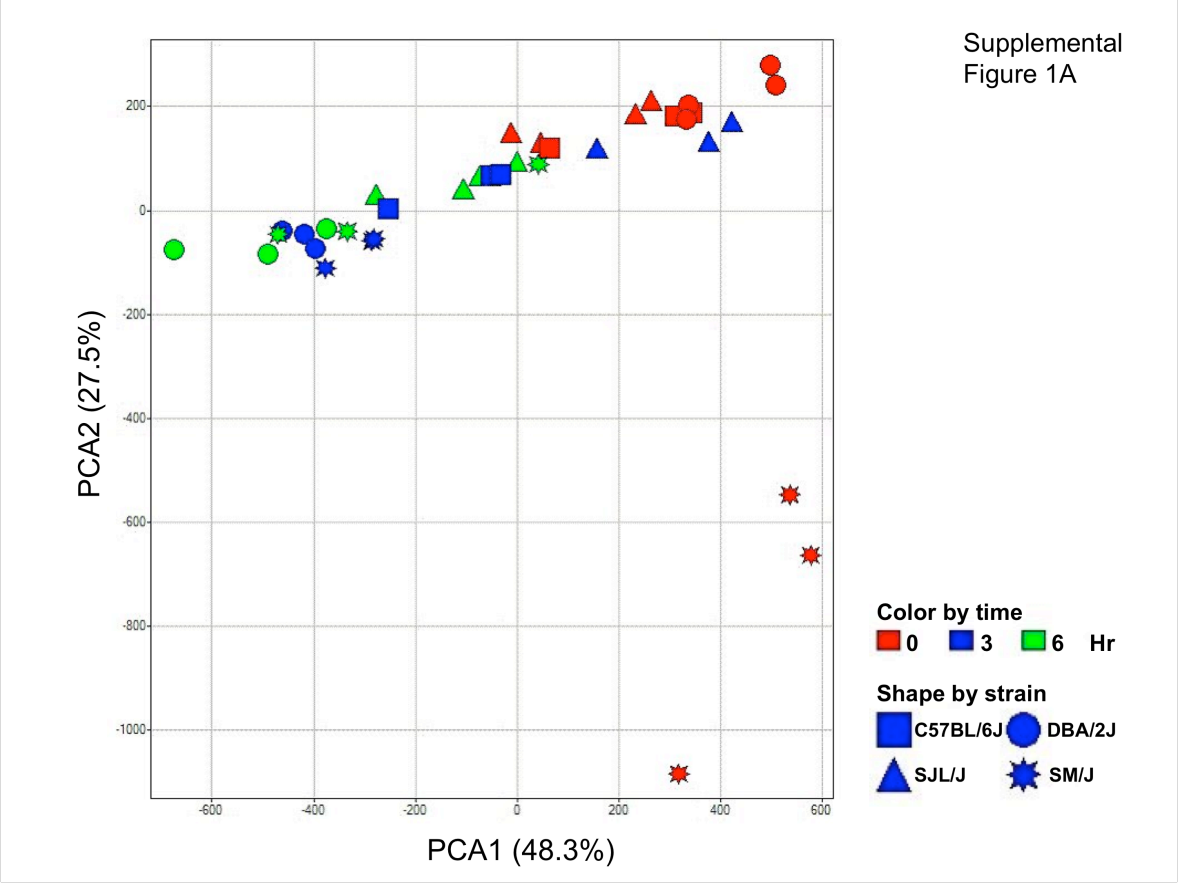
Supplemental Table 2. (*A*) The NMR chemical shifts and intensities for 10 metabolite peaks that differentiate the response of the resistant SJL/J strain from the 3 sensitive strains at 3 hours after acetaminophen treatment are shown. Mice from 4 inbred strains (SJL/J, C57BL/6J, SM/J and DBA/2J) were treated with a 300 mg/kg dose of acetaminophen. Livers were obtained at 0, 3 and 6 hrs after treatment and changes in endogenous metabolites were analyzed using ^{1}H-^{13}C 2-dimensional-NMR analysis (HSQC experiments) (Ulrich et al. 2008). The 400 peaks analyzed were filtered by criteria described in Supplemental Materials and Methods. Peaks meeting the criteria were ranked by the fold change between the maximal intensity versus the minimal intensity. Principal Component Analysis showed that the top 50 peaks were able to distinguish the response of resistant SJL/J mice from that of the 3 sensitive strains 3 hour after drug treatment. These 50 peaks were ranked by their ability to distinguish the 0 to 3 hr change in the

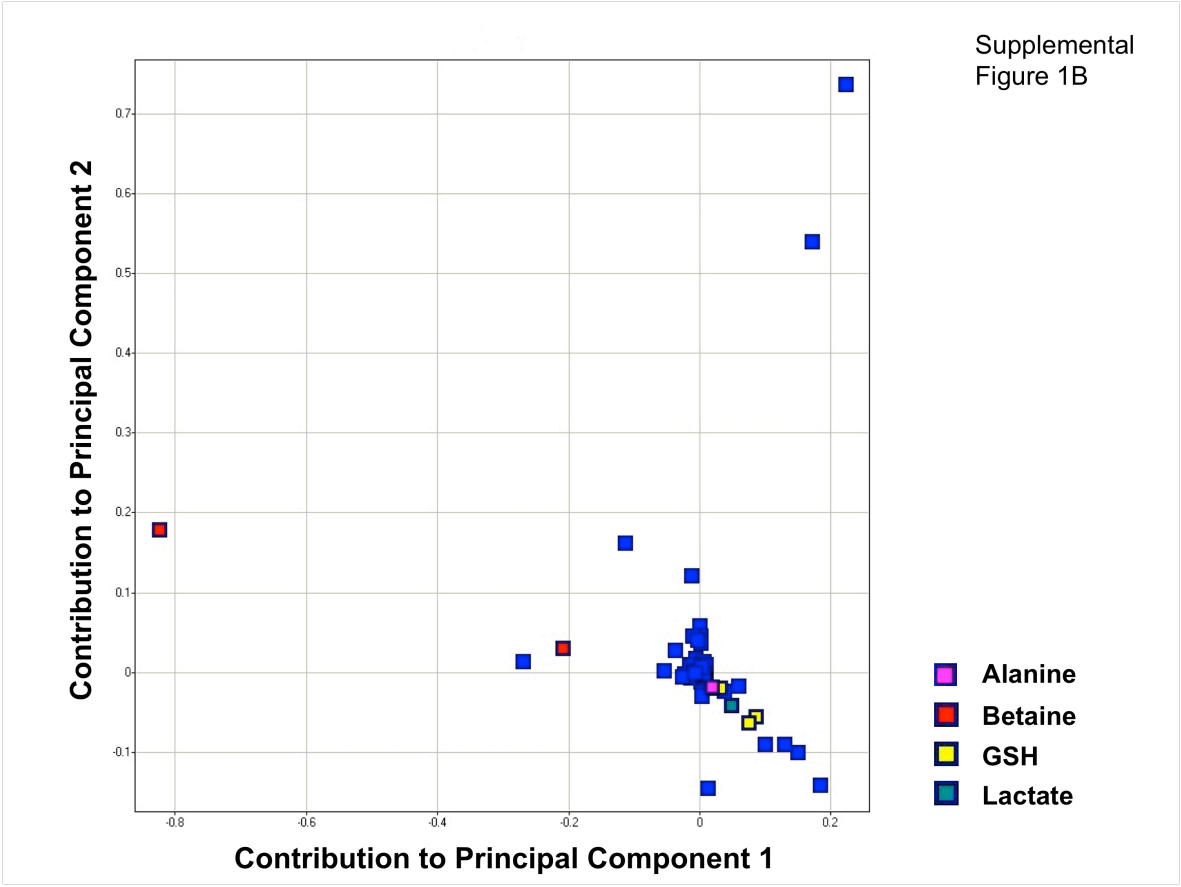
response of the resistant SJL/J mice from the other three strains. The top 10 peaks were identified. The intensity and ^{1}H and ^{13}C chemical shifts (ppm) are shown for each peak. The identities of 7 metabolite peaks were determined by comparing the observed chemical shifts with reference compound NMR data (Zheng et al. 2007) and confirmed by acquiring the spectra of samples spiked with reference compounds. The intensity of each peak is the average of three independent measurements. BDL indicates peaks that were below the level of detection, GSH is glutathione, and '???' indicates an unknown metabolite. (*B*) The contribution of the 50 metabolite peaks were used to construct the PCA plot to principal component 1 and 2. The peaks are ranked by their absolute contribution to the first component. The 10 peaks that best distinguish the metabolomic response of the resistant SJL/J strain from the other 3 susceptible strains at 3 hour after APAP dosing are highlighted in red. The peaks represented by question marks are from unknown metabolites.

Supplemental Table 3. (*A*) List of 224 genes whose expression profile was unique to SJL/J mice. Microarrays were used to analyze gene expression changes in liver tissue obtained 0 and 3 hours after treatment from 4 inbred strains with a 300 mg/kg dose of acetaminophen. Of the 35,000 transcripts analyzed, 224 had a drug-induced expression pattern that was significantly different (fold-change > 2 and p-value <0.01) in SJL/J mice relative to the 3 sensitive strains. Their functional annotation within the Gene Ontology database was analyzed, and 20 genes (**red bold text**) were annotated as involved in cellular metabolism. The enzymatic activity of three of these genes (*Pdk4*, *Bhmt2* and

G6pc) affected pathways involving the 4 endogenous metabolites identified by the metabolomic data. Each data point represents the average of three independent measurements. The standard deviation, present/absent call, fold change (FC), and calculated p-value are shown for each indicated strain and time point. (*B*) SYBR Green analysis of the level of hepatic *Bhmt2*, *G6pc*, and *Pdk4* gene expression in 4 inbred strains 3 hrs after a 300 mg/kg dose of acetaminophen. The expression level was normalized relative to that of β-Actin. Each measurement is the average of three replicates.

Supplemental Table 4. Gene expression data for 12 genes known to affect methionine, cysteine or glutathione biosynthesis. Microarrays were used to analyze gene expression changes in liver tissue obtained 0, 3 and 6 hrs after treatment of mice from 4 inbred strains (n=3 mice/time point) with a 300 mg/kg dose of acetaminophen. The level of expression of each gene, the standard deviation, the fold-change (relative to untreated), and the calculated p-value for the expression difference at the indicated time points are shown. *Bhmt2* was the only gene whose expression was uniquely altered in the resistant SJL/J strain (fold-change >2 and p-value<0.01) after acetaminophen treatment relative the 3 sensitive strains.


Supplemental
Figure 1A
PCA2 (27.5%)
PCA1 (48.3%)
200
0
-200
-400
-600
-800
-1000
-600
-400
-200
0
200
400
600
Color by time
0
3
6
Hr
Shape by strain
C57BL/6J
DBA/2J
SJL/J
SM/J


Supplemental
Figure 1B
Contribution to Principal Component 2
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.8
-0.6
-0.4
-0.2
0
0.2
Contribution to Principal Component 1
Alanine
Betaine
GSH
Lactate

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