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Obesity and cage environment modulate metabolism in the Zucker rat: a multiple biological matrix approach to characterising metabolic phenomena.

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Abstract

Obesity and its co-morbidities are increasing worldwide imposing a heavy socioeconomic burden. The effects of obesity on the metabolic profiles of tissues (liver, kidney, pancreas), urine and the systemic circulation were investigated in the Zucker rat model using ^{1}H NMR spectroscopy coupled to multivariate statistical analysis. The metabolic profiles of the obese (*fa*/*fa*) animals were clearly differentiated from the two phenotypically lean phenotypes, ((+/+) and (*fa*/+)) within

each biological compartment studied, and across all matrices combined. No significant differences were observed between the metabolic profiles of the genotypically distinct lean strains. Obese Zucker rats were characterized by higher relative concentrations of blood lipid species, cross-compartmental amino acids (particularly BCAAs), urinary and liver metabolites relating to the TCA cycle and glucose metabolism; and lower amounts of urinary gut microbial-host co-metabolites, and inter-matrix metabolites associated with creatine metabolism. Further to this, the obese Zucker rat metabotype was defined by significant metabolic alterations relating to disruptions in the metabolism of choline across all compartments analyzed. The cage environment was found to have a significant effect on urinary metabolites related to gut-microbial metabolism, with additional cage-microenvironment trends also observed in liver, kidney and pancreas. This study emphasises the value in metabotyping multiple biological matrices simultaneously to gain a better understanding of systemic perturbations in metabolism, and also underscores the need for control or evaluation of cage environment when designing and interpreting data from metabonomic studies in animal models.

Keywords: Zucker rat, cage effect, metabolic profiling, NMR spectroscopy

Introduction

Despite the growing global prevalence of obesity and related disorders encompassed by metabolic syndrome [1-3], many of the metabolic characteristics of obesity are poorly understood. Several genetic and environmental factors have been attributed as causal

in obesogenesis, yet questions regarding the mechanistic significance of the metabolic characteristics of obesity remain unanswered. In addition to host-related metabolism, the gut microbiota have been proposed to play a role in the development of obesity, dyslipidaemia and type 2 diabetes Mellitus (T2DM) but it is unclear as to the extent and nature of the contribution of the intestinal microbiota [4-5].

The Zucker rat is a widely used model for studying obesity and T2DM, as animals homozygous for the *fa* allele, a recessive missense point mutation in the leptin receptor, display significantly diminished sensitivity to leptin. As a result, these animals develop hyperleptinaemia, obesity, hyperphagia, hyperinsulinaemia, hyperlipidaemia, insulin-resistance and hyperglycaemia; sharing many classic signs of human metabolic syndrome [6]. As such, the Zucker rat may represent a useful tool to further understand the etiopathology of metabolic syndrome in humans.

The composition of blood and urine represents the sum of simultaneous metabolic processes and interactions occurring between various tissues and cell types in an animal. Thus, metabolic phenotyping of biofluids provides a snapshot of systemic metabolism, with profiling of blood and urine supplying complementary information. In addition, analysis of tissue samples can give valuable insights into the origin of the metabolic variation observed in biofluids and expand our understanding of the mechanistic processes associated with obesogenesis. The liver, as the organ responsible for very-low-density lipoprotein (VLDL) synthesis, is a key tissue in understanding the development of dyslipidaemia in the Zucker rat, with evidence of the dysregulation of lipid metabolism manifested in the blood. Furthermore, T2DM is

associated with renal complications whilst the disruption of pancreatic function is central to the pathology of diabetes [7-8].

Here, we used a ^{1}H NMR spectroscopy-based metabolic phenotyping approach to comprehensively characterize the global biochemical consequences of obesity and the metabolic syndrome across a total of five biological compartments (urine, blood, liver, kidney and pancreas). In animal experiments, individuals are often group housed and in many cases they are co-housed in treatment groups. Since small differences in the local environment can influence the phenotype of animals, the biological focus of the experiment can be confounded by study design [9-11]. In the current study tissues and biofluids were harvested from 14-week-old male obese *(fa/fa)*, and homozygous lean *(+/+)* and heterozygous lean *(fa/+)*, Zucker rats, with an animal husbandry arrangement designed to explore the effect of both obesity and cage microenvironment on the metabolic phenotype.

Materials and methods

Animal housing and sample collection

Male Zucker (*fa*/*fa, n* = 6) obese, lean (+/+, *n* = 7) and heterozygous lean (*fa*/+, *n* = 5) rats from the AstraZeneca colony were bred on site (Alderley Park, Cheshire, UK) from *fa*/+ parents, and housed in a conventional animal room in Techniplast P2000 cages on a 12h:12h light: dark cycle at standard room temperature and humidity. Pups were reared with their mothers until they were weaned and then housed as littermates in six cages, each containing one rat from each genotype (*n* = 3 per cage), apart from cage two, which was found to contain an obese and two (+/+) rats, following

genotype verification. The housing arrangement described here was used in order to minimize cage effects seen in our previous study[12] that may have resulted from the co-housing of different strains separately rather than genotypic effects on the gut microbiota. Each of the six cages had different parents. Food (SDS breeding diet RM-3) and water were available *ad libitum* throughout the study. At weekly intervals, from 5 to 14 weeks of age, the animals were transferred to a procedures room and weighed (weight data shown in supplementary Figure S1). Urine was collected at 14 weeks of age by placing the animals individually in metabolism cages, for no more than 2 hours. Urine was stored at -20 °C, until analysis. The rats had access to food and water whilst in the metabolism cages. At 14 weeks of age animals were rendered insentient by inhalation of a 5:1 mixture of CO_2:O_2 and a blood sample was taken by cardiac puncture into lithium heparin blood syringes and centrifuged at 2400 *g* for 10 minutes. The plasma was then removed and stored at -20 °C until analysis. Liver, kidney and pancreas tissues were removed and snap frozen in liquid nitrogen. Samples were stored at -40 °C prior to analysis. Euthanasia was confirmed by cervical dislocation. All animal work was carried out in accordance with the U.K. Home Office Animals (Scientific Procedures) Act 1986 under a Project Licence approved by the AstraZeneca Ethical Review Committee. The specific protocols described in this paper were also reviewed and approved by the local Departmental Review to ensure that they adhered to the principals of minimising animal suffering.

^{1}H NMR spectroscopy of tissues, plasma and urine

Plasma samples were thawed at room temperature and mixed by vortexing, then 100 µL was combined with 450 µL of saline solution (0.9% NaCl w/v, in H_2O:D_2O 8:2). Urine samples were prepared by combining 400 µL of urine with 200 µL of phosphate

buffer (pH 7.4), prepared in 8:2 H_2O:D_2O, containing 1mM 3-trimethylsilyl-1-[2,2,3,3,-2H4] propionate (TSP) as a chemical shift reference and 3 mM of sodium azide as a bacteriostatic agent. The mixture was vortexed and centrifuged at 16000 *g* for 10 minutes, before 500 µL or 550 µL of the supernatant was transferred to a 5 mm outer diameter NMR tube, for plasma and urine samples, respectively.

Aqueous extracts were prepared from liver, kidney and pancreatic tissue by combining tissue (~60 mg) with 600 µL ice-cold $CHCl_3$:MeOH (2:1 V/V) in a 2 ml Eppendorf tube. For each tissue, samples were removed from the same anatomical location for each animal; for the kidney this incorporated both the medulla and cortex. Samples were immediately homogenized using a TissueLyser from Qiagen (West Sussex, UK), with one 5 mm stainless steel bead per sample, for 8 minutes at 25 Hz. The homogenate was combined with 600 µL H_2O, vortexed to mix, and left on ice for 10 minutes. Samples were centrifuged at 16000 *g* for 10 minutes and the upper aqueous layer of supernatant was collected. To increase metabolite recovery, a second extraction was performed on the sample; the sample pellet was resuspended in 600 µL ice-cold $CHCl_3$:MeOH (2:1 V/V), vortexed, and left on ice for 10 minutes, before centrifugation at 16000 *g* for 10 minutes. The aqueous layer of supernatant was again collected and combined with the first aqueous extraction [13]. Solvents were removed from the aqueous extract by speed vacuum concentration using an Eppendorf Concentrator plus. Samples were stored at -40 °C until the day of ^{1}H NMR analysis. On the day of analysis, 700 µL of D_2O:H_2O (9:1 V/V), containing 1 mM TSP as a chemical shift reference, were added to each sample, and vortexed to ensure reconstitution. Samples were centrifuged at 16000 *g* for 10 minutes, before 550 µL of the supernatant was transferred to a 5 mm outer diameter NMR tube.

Plasma ^{1}H NMR spectra were acquired using a 600 MHz Bruker Avance III-600 spectrometer (Rheinstetten, Germany) with a 5 mm TCI probe and CryoProbe system operating at 600.13 MHz ^{1}H frequency. The plasma spectra were acquired using the Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence (RD-90°-(t-180°-t)n-acquire FID)[13], with irradiation of the water peak during the RD, in order to attenuate the broad signals from proteins, lipoproteins and other high molecular weight compounds [14-15]. The 90° pulse length was adjusted for each sample individually.

^{1}H NMR spectra of urine and aqueous tissue extracts were acquired using a 600 MHz Bruker Avance DRX600 spectrometer (Rheinstetten, Germany) with a 5 mm BBI probe and TXI probe for liver and kidney, pancreas and urine samples, respectively. A standard one-dimensional pulse sequence was used: RD-90°-t-90°-tm-90°-acquire free induction decay (FID) [t = 3 μs]. The water resonance was selectively irradiated during the relaxation delay (RD) of 2 s and again during the mixing time (tm) of 100 ms. The 90° pulse length was adjusted to 15.25 μs for urine, 10.68 μs for liver tissue extracts, and 10.88 μs for kidney and pancreas. For acquisition of both plasma and tissue spectra, the temperature was kept constant at 300 K, the field frequency was locked on D_2O solvent and 128 scans were recorded into 64k data points.

Acquired ^{1}H NMR spectra were manually corrected for phase and baseline distortions; plasma ^{1}H chemical shifts were referenced internally to the α-glucose H_1 resonance at δ 5.233, whereas urine and aqueous tissue extracts were referenced to the internal standard, TSP, at δ 0.0, using TOPSPIN (version 3.1, Bruker BioSpin). The spectra were exported into MATLAB (MathWorks) and digitised using a script

developed in-house (https://csmsoftware.github.io/docs/impacts/index.html). The spectral regions containing resonances from water [δ 4.6-5.2] and TSP were excised prior to statistical analysis. The aqueous pancreas extracts of five samples contained lipid resonances; these spectral regions were set to zero integral in all sample spectra. All spectra were aligned [16] and the tissue spectra normalized to the probabilistic quotient to partially compensate for differences in total sample volumes of tissue extracts [17]. Molecules were assigned with the aid of a combination of two-dimensional homonuclear NMR spectroscopy (J-resolved spectroscopy, correlation spectroscopy, total correlation spectroscopy), statistical total correlation spectroscopy[18] and an in house database built from authentic standards.

Data analysis strategy

Multivariate statistical analysis

The spectral data were imported into SIMCA 12.0 (Umetrics 2009); PCA was used as an initial unsupervised multivariate statistical method to gain an overview of the inter-sample variation and to identify outliers [19]. OPLS and OPLS-DA were used to detect the maximal differences in metabolic profiles between the three differing genotypes. Supervised models were constructed in MATLAB using a procedure developed in-house [20], using ^{1}H NMR spectral data as the descriptor matrix and genotype as the response variable (Y predictor). Pairwise OPLS-DA models were constructed for the plasma and each tissue, comparing the samples from each of the three genotypes. Seven-fold cross validation was used to obtain cross-validated scores. The significance of the predictive value of each supervised model was validated by permutation testing, wherein the Y matrix of the model was permuted 1000 times, using a script in MATLAB [21].

Univariate statistical analysis

Univariate statistical analysis was performed using R and Python. The loadings plot of each OPLS-DA model was used to select which molecules to subject to two-way ANOVA; the Benjamini-Hochberg false discovery rate (FDR)-corrected significant correlation coefficient cut-off was calculated for each model, and regions with an r^2 above this value were selected for further analysis. Representative integral regions for each metabolite were compared using a two-way ANOVA with genotype and cage as factors. A Levene's test was used to assess equality of variances and the data transformed where necessary. A Tukey's range test was used for post-hoc analysis. Finally, to address the issue of multiple comparisons, for each metabolic compartment, the spectra were binned into ~3000 bins, and a two-way ANOVA performed on each bin with p-values adjusted using the Benjamini-Hochberg correction.

Multi-compartment clustering analysis

Unsupervised hierarchical clustering analysis (HCA) was performed to compare all animals across all the biological matrices analyzed and determine any patterns of correlation between metabolites. Metabolites were selected for inclusion using the key discriminatory metabolites identified through pairwise OPLS-DA genotypic comparison models and the metabolites associated with significant cage-related variation, ascertained using two-way ANOVA. Integral data were first standardised as *z*-scores, such that the mean was 0 and the SD was 1 for each metabolite. Spearman's rank correlation for similarity measurement and Ward's linkage for clustering were

used for the unsupervised HCA. The functions hclust and heatmap.2 were used to generate the HCA and heatmap in R.

Results

Adiposity alters the urinary metabolite profile

PCA of the urine spectra indicated a clear phenotypic trend and the presence of a metabolic outlier, the *fa/fa* rat from cage 1 (F1), Figure 1A. The predictive ability of the OPLS-DA model comparing homozygous animals was hindered by the influence of the F1 animal ($Q^2Y = 0.41$, supplementary Figure S2) with the exclusion of this animal illustrating this effect (resultant improvement in prediction $Q^2Y = 0.73$, Figure 1F). The OPLS-DA comparison of obese and heterozygous lean animals yielded a model with poorer predictive ability ($Q^2Y = 0.21$, supplementary Figure S3), which showed even greater improvement upon removal of the F1 animal ($Q^2Y = 0.78$, supplementary Figure S4). OPLS-DA loadings plots comparing obese and lean animals identified the key discriminatory metabolites to be hippurate, 3-indoxyl sulphate, phenylacetylglycine (PAG), methylamine, creatinine, 4-guanidinobutanoic acid and *N*-acetyl glycoprotein, which were all increased in lean, relative to obese animals, and trigonelline, 2-oxoglutarate, formate, fumarate, glucoronate and hypotaurine, which were all higher in the urine from obese rats (Figure 1F). A summary of the metabolite differences in the obese animals relative to the lean animals can be found in Table 1.

Comparison of the two lean genotypes using OPLS-DA resulted in a model that failed permutation testing, reflecting limited systematic variation between these two groups.

Adiposity modulates the systemic metabolic phenotype

Phenotype was the strongest source of variance in the PCA model constructed from the plasma metabolic profiles (Figure 1B). Greater amounts of lipid and branched chain amino acids (BCAAs) were observed in the plasma from obese animals, underlying the variation described by the first principal component. The obese animal from cage one was an outlier in this model, due to much higher concentrations of plasma lipid species, and thus the sample was excluded from the supervised discriminant analysis to aid interpretation of results.

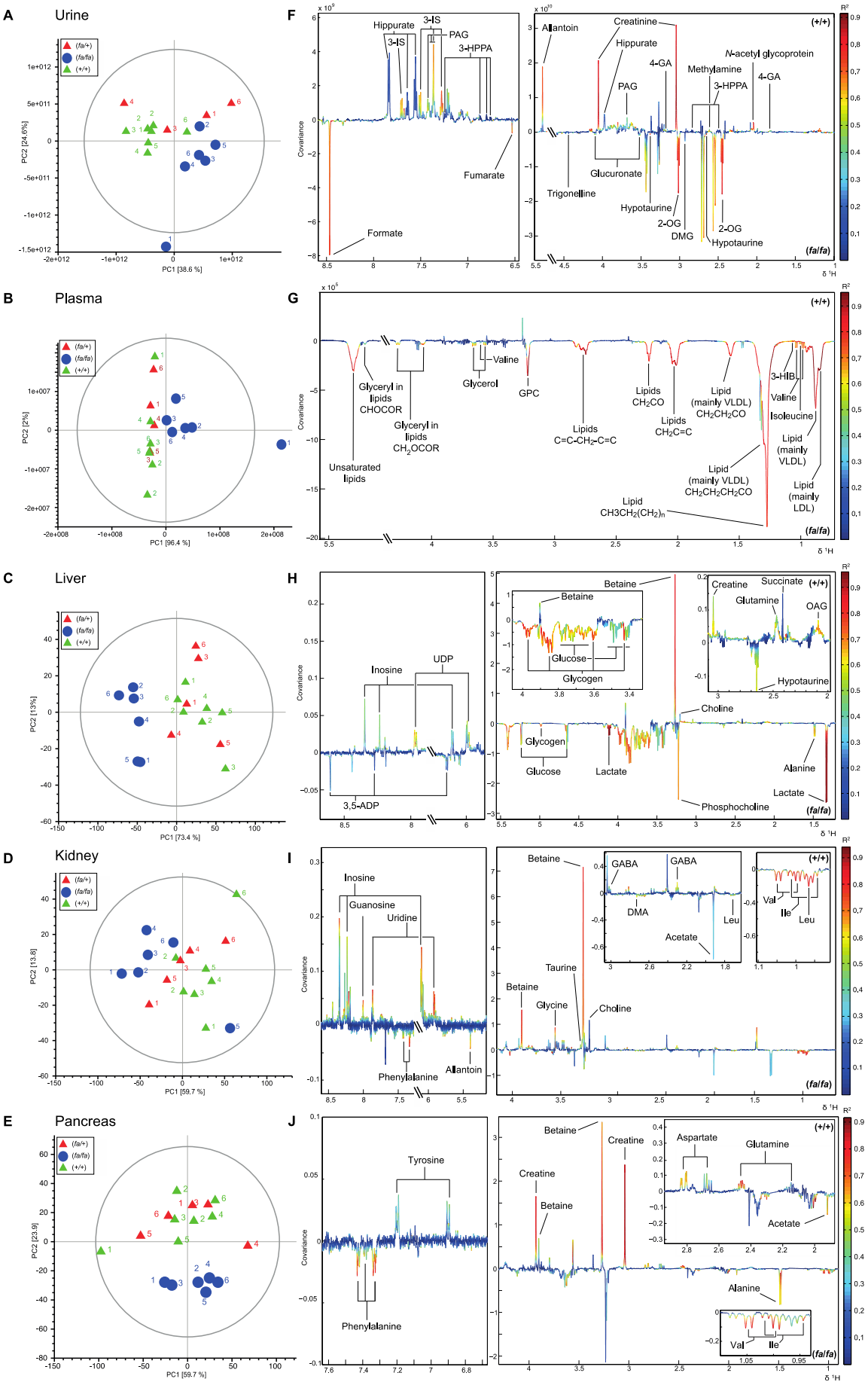
A
Urine
B
Plasma
C
Liver
D
Kidney
E
Pancreas
F
G
H
I
J
(fa/+)
(fa/fa)
(+/+)
PC1 [38.6 %]
PC2 [24.6%]
PC1 [96.4 %]
PC2 [2%]
PC1 [73.4 %]
PC2 [13%]
PC1 [59.7 %]
PC2 [13.8]
PC1 [59.7 %]
PC2 [23.9]
Covariance
δ ¹H
R²
Hippurate
3-IS
PAG
3-HPPA
Fumarate
Formate
Allantoin
Creatinine
Hippurate
4-GA
N-acetyl glycoprotein
Methylamine
3-HPPA
PAG
Trigonelline
Glucuronate
Hypotaurine
2-OG
DMG
Glyceryl in lipids CHOCOR
Glyceryl in lipids CH2OCOR
Unsaturated lipids
Glycerol
Valine
GPC
Lipids C=C-CH2-C=C
Lipids CH2CO
Lipids CH2C=C
Lipid (mainly VLDL) CH2CH2CO
Lipid (mainly VLDL) CH2CH2CH2CO
Lipid CH3CH2(CH2)n
3-HIB
Isoleucine
Lipid (mainly VLDL)
Lipid (mainly LDL)
Inosine
UDP
3,5-ADP
Betaine
Glucose
Glycogen
Creatine
Succinate
Glutamine
OAG
Hypotaurine
Choline
Lactate
Alanine
Phosphocholine
Guanosine
Uridine
GABA
DMA
Acetate
Leu
Val
Ile
Taurine
Glycine
Phenylalanine
Tyrosine
Aspartate

Figure 1: PCA scores plots of all genotypes (A-E) and OPLS-DA coefficient loadings plots comparing homozygous lean and obese Zucker rats (F-J) for each biological matrix. Model statistics: A: 3 principal components, Y= 0.35, R^2 = 0.77; B: 2 principal components, Q^2Y = 0.97, R^2 = 0.98; C: 2 principal components, Q^2Y = 0.81, R^2 = 0.86; D: 5 principal components, Q^2Y = 0.80, R^2 = 0.93; E: 2 principal components, Q^2Y = 0.77, R^2 = 0.84; F: Q^2Y = 0.73, R^2 = 0.86, 1 predictive, 0 orthogonal components; G: Q^2Y = 0.8, R^2 = 0.89, 1 predictive, 1 orthogonal component; H: Q^2Y = 0.78, R^2 = 0.90, 1 predictive, 0 orthogonal components; I: Q^2Y = 0.69, R^2 = 0.92, 1 predictive, 0 orthogonal components; J: Q^2Y = 0.49, R^2 = 0.97, 1 predictive, 0 orthogonal components. The strain of the animal is indicated by colour and the numbers shown indicate the cage number (A-E). 2-OG, 2-oxoglutarate; 3-HIB, 3-hydroxyisobutyrate; 3-HPPA, m-hydroxyphenylpropionate; 3-IS, 3-Indoxyl sulphate; 3,5-ADP, adenosine 3',5'-diphosphate; 4-GA, 4-guanidinobutanoic acid; DMA, dimethylamine; DMG, dimethylglycine; GABA, gamma amino butyric acid; GPC, glycerophosphorylcholine; Ile, isoleucine; Leu, leucine ; OAG, O-acetyl glycoprotein; PAG, phenylacetylglycine; ppm, parts per million; UDP , uridine diphosphate; Val, valine.

OPLS-DA models with good predictive ability (Q^2Y = 0.8) were obtained by comparing the obese plasma metabolic profiles with those from the homozygous (+/+) lean genotypes (Figure 1G). The most significant discriminatory metabolites were found to be increased lipid species, including unsaturated lipids, and resonances from lipoproteins (VLDL and LDL); glycerol; glycerophosphocholine (GPC); 3-hydroxyisobutyrate (3-HIB); and the branched-chain amino acids (BCAAs), valine and isoleucine; in the obese (*fa/fa*) samples compared to the lean samples. The OPLS-DA model comparing the heterozygous lean and obese plasma profiles also had good predictive ability (Q^2Y = 0.67) and can be found in the supplementary information (Figure S5).

No separation was observed between the (*fa*/+) and (+/+) strains in the PCA scores plot, indicating that genotype did not affect the plasma metabolic signatures of the lean animals. This was confirmed by the poor predictive performance of the OPLS-DA model built on these plasma metabotypes, which failed permutation testing.

Table 1. Summary of two-way ANOVA results for all biological matrices

			Significant genotypic variation observed			*Significant cage-associated effect observed*
Molecule	^{1}H (ppm) (multiplicity)	Tissue/biofluid	*fa/fa* vs +/+	*fa/fa* vs *fa/+*	+/+ vs *fa/+*	Tissue/biofluid
Amino acid metabolism						
3-hydroxyisobutyrate	1.06(d), 2.48(m), 3.53(dd), 3.69(dd)	**Blood**	**	**		
Aspartate	2.69(dd), 2.80(dd), 3.89(dd)	**Pancreas**	**	*		
Glutamine	2.14(m), 2.46(m), 3.78(t)	**Liver**	**			
		Pancreas	**	**		
4-guanidinobutanoic acid	1.81(q), 2.24(t), 3.17(t)	**Urine**	**	**		
Isoleucine	0.94(t), 1.01(d), 1.26(m), 1.48(m), 1.98(m), 3.68(d)	**Blood**	**	**		
		Kidney	***	**		
		Pancreas	*			
Leucine	0.95(t), 1.71(m), 3.73(t) or 0.94(d), 0.96(d), 1.71(m), 3.73(t)	**Kidney**	***	**		
Phenylalanine	3.13(dd), 3.28(dd), 4.0(dd), 7.34(d), 7.39(m), 7.44(m)	**Kidney**	***	**		
		Pancreas	***	**		
Tyrosine	3.06(dd), 3.20(dd), 3.94(dd), 6.89(d), 7.18(d)	Pancreas	*	**		Pancreas
Valine	0.99(d), 1.04(d), 2.28(m), 3.62(d)	**Blood**	***	**		
		Kidney	***	**		
		Pancreas	**	*		
Taurine metabolism						
Hypotaurine	2.66(t), 3.37(t)	Liver	*	*		
		Urine	*			
Taurine	3.27(t), 3.43(t)	Kidney		**	*	
Gut microbial-host co-metabolism						
3-HPPA	2.48(t), 2.84(t), 6.76(d), 6.80(s), 6.92(dd), 7.27 (t)					**Urine**
3-indoxyl suphate	7.21(dd), 7.28(dd), 7.38(s), 7.51(d), 7.70(d)	**Urine**	*	*		
Dimethylglycine	2.92(s), 3.72(s)	**Urine**			*	Urine
Hippurate	3.97(d), 7.55(t), 7.64(t), 7.84(d)	**Urine**	**	**		**Urine**
Phenylacetylglycine	3.67(s), 3.75(d), 7.35(t), 7.35(d), 7.42(t)	**Urine**	*			
Trigonelline	4.43(s), 8.08(m), 8.91(m), 9.11(s)	**Urine**	*			Urine
Choline metabolism						
Betaine	3.26(s), 3.90(s)	**Liver**	***	**		
		Kidney	*			
		Pancreas	**	**		
Choline	3.21(s), 3.52(m), 4.07(m)	**Liver**	*	*		Kidney, Liver
Dimethylamine	2.72(s)	Kidney	**	**		
Glycerophosphocholine	3.23(s), 3.61(dd), 3.68 (t), 3.72(dd), 3.90(m), 4.32(t broad)	**Blood**	****	***		
Methylamine	2.61(s)	Urine	*	*		
Phosphocholine	3.22(s), 3.60(t), 4.18(dd)	**Liver**	**	**		
Nucleoside/purine/pyramidine metabolism						
3,5-ADP	4.52(bs), 6.16 (d), 8.25(s), 8.58(s)	**Liver**	**	***		Liver
Allantoin	5.38(s)	**Kidney**	***	**		
		Urine	**	*		
Guanosine	3.86(qd), 4.24(q), 4.41(t), 5.91(d), 8.00(s)	**Kidney**	***	*		
Inosine	3.85(dd), 3.92(dd), 4.28(q), 4.44(t), 6.10(d), 8.24(s), 8.34(s)	**Kidney**	**			
		Liver	*			
Uridine	3.81(dd), 3.92(dd), 4.14(q), 4.24(t), 4.36(t), 5.90(s), 5.92(d), 7.87(d)	**Kidney**	*			
Uridine diphospate	4.21(dd), 4.25(dd), 4.37(dt), 4.39(dd), 4.43, 5.96(m), 5.98(d), 7.98(d)	**Liver**	**	**		
Creatine metabolism						
Creatine	3.03(s), 3.94(s)	Liver	*			
		Pancreas	****	***		
Creatinine	3.05(s), 4.06(s)	**Urine**	***	***		
Glycine	3.56(s)	**Kidney**	***	*		Kidney
TCA cycle/energy metabolism						
2-oxoglutarate	2.45(t), 3.01(t)	**Urine**	**		*	
Acetate	1.92(s)	Kidney	*			
		Pancreas	**		*	Kidney
Alanine	1.48(d), 3.79(q)	**Liver**	***	***		
		Pancreas	***	**		Liver
Formate	8.46(s)	**Urine**	**	*		
Fumarate	6.53(s)	**Urine**	**			
Glucose	α-glucose: 3.42(t), 3.54(dd), 3.71(t), 3.83(ddd), 3.84(m), 3.76(m), 5.22(d); β-glucose: 3.24(dd), 3.40(t), 3.47(ddd), 3.48(t), 3.72(dd), 3.90(dd), 4.65(d)	**Liver**	**	*		
Glucuronate	3.29(m), 3.50(m), 3.58(dd), 3.73(m), 4.08(d), 4.65(d), 5.25(d)	Urine	*			
Glycogen	5.38-5.45(m)	**Liver**	***	**		
Lactate	1.33(d), 4.11(q)	**Liver**	****	***		
Succinate	2.41(s)					Liver
Dyslipidaemia						
Glycerol	3.56(dd), 3.65(dd), 3.78(m)	Blood	*			
C_3H in glycerol backbone	5.2(bs)	**Blood**		*		
$>C_3H_2$ in glycerol backbone	4.07(bs)	**Blood**		*		
Lipid [$-CH_2CH_2N^+(CH_3)$]	4.27(bs)	**Blood**		*		
Lipid [$-CO-CH_2CH_2-$]	1.57(bs)	**Blood**	***	***		
$-CH_3$ (LDL)	0.85(bs)	**Blood**	****	****		
Lipid [$CH_2HC=$]	2.01(bs)	**Blood**	***	***		
Lipid [$-CO-CH_2-$]	2.32(bs)	**Blood**	***	***		
Lipid [$=CHCH_2CH=$]	2.75(bs)	**Blood**	****	***		
$-CH_3$ (VLDL)	0.88(bs)	**Blood**	****	****		
Lipid [$-(CH_2)_n-$]	1.27(bs)	**Blood**	****	****		
Lipid [-HC=CH-]	5.3(bs)	**Blood**	****	****		
Miscellaneous						
GABA	1.91(q), 2.30(t), 3.02(t)	**Kidney**	*			
N-acetyl glycoprotein	2.04(m)	**Urine**	***	***		

Colour of matrix indicates the genotype observed to have the highest relative concentration *(fa/fa*, blue; +/+, green, *fa/+,* red). Abbreviations: s, singlet; d, doublet; dd, double of doublets; t, triplet; bs, broad singlet; m, multiplet; q, quartet. Statistical significance of genotypic comparisons derived from the Tukey's range test post-hoc analysis is indicated: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** < 0.0001; biological matrix is shown in bold where significance passes FDR. Statistical significance for post-hoc analyses of cage variation can be found in figure 2**.**

Altered hepatic metabolism associated with obesity

As with the plasma, PCA analysis identified phenotype as the strongest source of variation in the liver metabolic profiles, described by principal component (PC) 1 (Figure 1C), with no obvious genotype-associated distinction between the lean strains. Pairwise OPLS-DA comparisons of the obese hepatic profiles with either the homozygous or heterozygous lean profiles returned models with strong predictive ability (Q^2Y = 0.78 and 0.70, respectively). Samples from obese animals were characterized by higher concentrations of lactate, alanine, hypotaurine, phosphocholine, glycogen, glucose and adenosine 3', 5'-diphosphate (3,5-ADP), and lower concentrations of glutamine, *O*-acetylglycoproteins, creatine, choline, betaine, inosine and uridine diphospate (UDP), compared to their lean equivalents (Figure 1H (*fa/fa* versus +/+); Supplementary Figure S6 (*fa*/+, *fa*/*fa*)).

Renal metabolic characteristics of obesity

The PCA model constructed from the renal metabolic phenotypes identified an obese animal (cage five) as an outlier. The sample occupied a metabolic space separate from both the obese and lean metabolite phenotypes in the PCA scores, with similarities to both metabolic phenotypes, and also a greater relative concentration of phosphocholine compared to all the other samples (Figure 1D). This sample was excluded from subsequent supervised multivariate analyses, to aid interpretation of phenotypic variation. Despite slight genotypic clustering of the lean samples in the

PCA scores, further supervised comparison of the two lean genotypes found no predictable variation between the two groups, as judged by permutation testing, indicating a negligible effect of genotype on the lean kidney metabolite profile.

Pairwise OPLS-DA comparisons of the obese renal metabolic profiles with those of the homozygous (+/+) animals (Figure 1I; $Q^2Y = 0.69$) identified clear metabolic differences. Obese-derived kidney tissue was observed to contain greater amounts of BCAAs (valine, isoleucine and leucine), phenylalanine, acetate, allantoin and dimethylamine (DMA) compared to their lean counterparts and lower alanine, betaine, glycine, uridine, inosine, guanosine and gamma-aminobutyric acid (GABA).

The model comparing obese and heterozygous lean animals had poor predictive ability (supplementary Figure S7; $Q^2Y = 0.37$), which may reflect greater variability in the heterozygous, compared to homozygous, kidney samples.

Obesity associated pancreatic metabolic signature

PCA demonstrated a clear pattern of phenotypic clustering, despite differences within the lean samples being the strongest source of variation (Figure 1E). An OPLS-DA model with moderate predictive ability was obtained comparing the obese pancreatic metabolic profiles with those of the homozygous lean animals ($Q^2Y = 0.49$). Pancreatic tissue from the obese animals was found to contain higher amounts of valine, isoleucine, alanine, acetate and phenylalanine than that of lean animals, and lower amounts of aspartate, creatine, betaine, glutamine and tyrosine (Figure 1J).

The OPLS-model constructed comparing the obese with the heterozygous lean animal pancreatic profiles was not found to be robust, failing permutation testing. As with the kidney analysis, this result may reflect higher variability in the heterozygous samples. No predictive systematic variation between the two lean genotypes was detected using OPLS-DA.

Cage environment impacts urine and tissue metabolite profiles

Two-way ANOVA was employed to evaluate the effect of both genotype and cage on the relative metabolite abundances measured, with significant cage-associated differences observed in urine for the host-gut microbial co-metabolites *m*-hydroxyphenylpropionate (3-HPPA) and hippurate, and trends in dimethylglycine and trigonelline measurements. Cage-associated trends were also observed for all three tissue extracts, with effects observed in hepatic alanine, choline, 3,5-ADP and succinate; renal glycine, choline, and acetate; and pancreatic tyrosine (Figure 2). A summary of the results of the two-way ANOVA analyses can be found in Table 1.

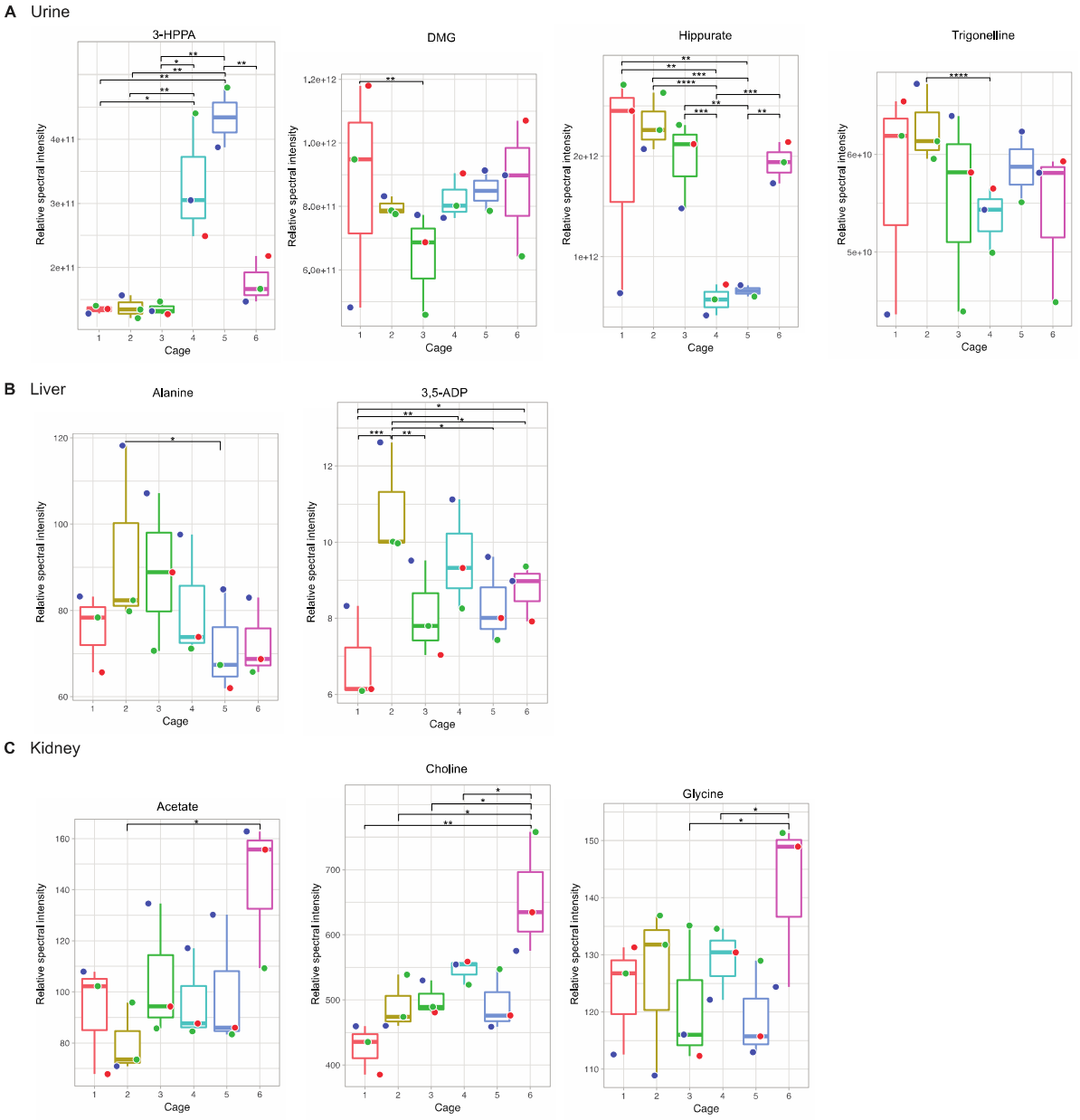

Figure 2: Boxplots of metabolites observed to have significant cage-associated variation in urine (A), liver (B) and kidney (C) samples. Results from pairwise comparisons performed using the Tukey's range test post-hoc analysis is indicated: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** < 0.0001. The colour of each data point represents the genotype of the animal *(fa/fa*, blue; +/+, green, *fa/+,* red).

Multi-compartmental impact of obesity on metabolism

The unsupervised HCA performed demonstrated clear cross-matrix phenotypic trends, with no genotypic clustering evident within the lean phenotype (Figure 3). Additionally, the outlier obese animal, identified in both urine and blood OPLS-DA models (F1), was clearly described by the cluster analysis. Obese Zucker rats were

characterized by higher relative concentrations of blood lipids, as well as cross-compartmental amino acids, particularly BCAAs, and metabolites relating to the TCA cycle and glucose metabolism. The obese animals were also characterized by lower amounts of urinary metabolites of gut microbial-host co-metabolic origin, and inter-matrix metabolites pertaining to creatine metabolism.

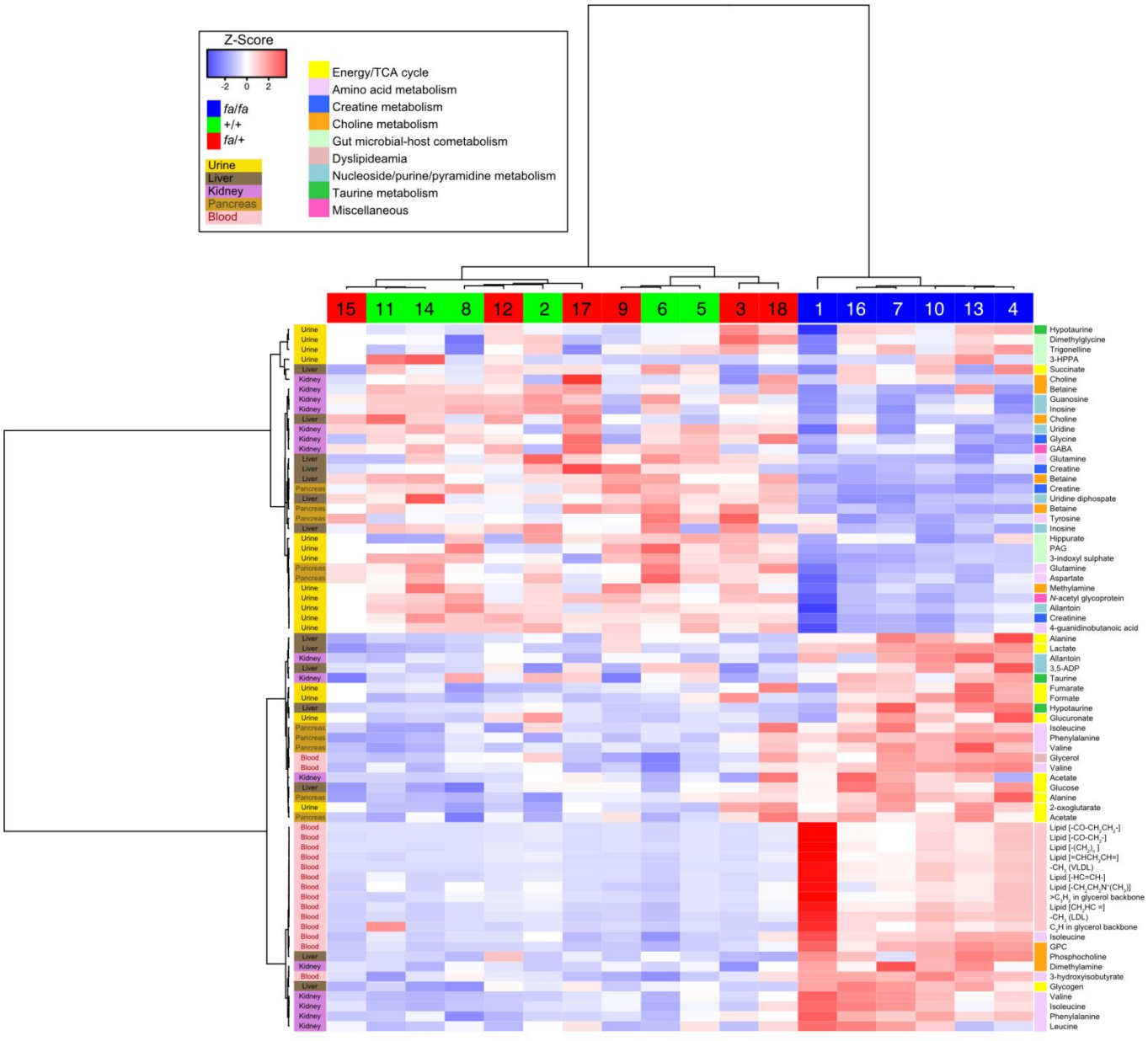

Figure 3: Dendrogram generated from HCA of *z*-score standardized metabolite integrals. *Z*-scores are shown as a heatmap; shades of red and blue represent higher and lower values, respectively, compared with the mean. Columns represent each individual animal (numbered 1-18), coloured according to the genotype of the animal (*fa/fa*, blue; +/+, green, *fa/+,* red). Each row represents a metabolite from a single biological matrix, with the biological matrix indicated and colour-coded on the left, and the related metabolic pathway/pathology indicated

by colour coding on the right. 3-HPPA, m-hydroxyphenylpropionate; 3,5-ADP, adenosine 3',5'-diphosphate; GABA, gamma amino butyric acid; GPC, glycerophosphorylcholine; PAG, phenylacetylglycine.

Discussion

Using a ^{1}H NMR spectroscopy-based metabolic phenotyping approach, the biochemical perturbations associated with obesity have been investigated by characterization of the metabolic profiles of urine, plasma and organs known to be affected by obesity. As expected, obesity resulted in modulations in lipid and energy metabolism, with alterations in choline, amino acid, creatine, nucleoside and microbial-host co-metabolism also observed. In addition, the cage environment was found to have a significant influence on certain urinary metabolites predominantly relating to gut-microbial metabolism, with cage-microenvironment trends also observed in liver, kidney and pancreas.

A significantly greater excretion of 2-oxoglurate and fumarate, and a non-significant trend of higher urinary citrate, was observed in the obese Zucker rats compared to their homozygous lean equivalents, suggesting up-regulation of the TCA cycle. This is consistent with previous investigations of leptin mutation-derived rodent models of obesity [12, 22-24].

The leptin receptor mutation present in the obese Zucker rat causes hyperphagia, with obese rats consuming approximately 30-50% more food than their lean littermates [25-28]. However, there is evidence to suggest that hyperphagia is not the sole cause of hyperlipidaemia and hepatic fat deposition in this rodent model, and that obesity in this model is due to an abnormal pattern of energy utilization, with a lower rate of

protein deposition and heat production, and a higher rate of fat deposition [29-30]. Thus, it is likely that the differences in caloric intake, as well as in energy expenditure and utilization, between the obese and lean rats, influenced the differences in TCA cycle intermediates observed here.

In addition to phenotypic variation associated with urinary TCA cycle metabolites, the obese animals had higher urinary formate, relative to the lean animals. Formate, the simplest carboxylic acid, is an intermediate of several metabolic processes, playing a key role in one-carbon metabolism [31]. As a by-product of microbial dietary fiber fermentation in the gut, and a metabolite utilized in bacterial cross-feeding, differences in formate excretion observed here could reflect variation in the functional activities of the intestinal microbiota [32-35].

The observation of increased hepatic glycogen in the obese animals is indicative of dysregulated glucose metabolism, and together with the findings relating to hepatic glucose, lactate and alanine, most likely relates to the development of insulin resistance in the obese rats [36]. While the obese Zucker rat has been widely used as a model of genetic obesity, it has not generally been used as a model of T2DM; studies have shown the animals to be relatively normoglycemic or only marginally hyperglycemic [36-37], but with abnormal glucose tolerance [38-39]. However, obese Zucker rats are hyperinsulinemic [40] and show significant hepatic as well as peripheral insulin resistance [41], which is established by approximately 7-13 weeks of age [38, 42-43].

Several metabolic differences between obese and lean animals were indicative of altered glucose catabolism and storage in the obese animals, including increased

hepatic glucose, glycogen and lactate, urinary glucuronate, and hepatic and pancreatic alanine. Abnormal hepatic carbohydrate metabolism has previously been observed in the obese Zucker rat [44], with increased hepatic glucose produced from non-carbohydrate sources. Increased hepatic glucose has been observed in non-insulin dependent diabetes mellitus patients, with more than 80% of the increased hepatic glucose attributed to increased gluconeogenesis [45].

Hepatic lactate and alanine were significantly higher in the obese rats, compared to the lean, and clustered together in the HCA performed. These findings are consistent with previous analyses of the obese Zucker liver [46-47], a hyperlipidemic hamster model [48] and in a high-fat feeding-induced mouse model of insulin resistance [49]. Additionally, increased hepatic hyperpolarized [1-^{13}C]lactate and [1-^{13}C]alanine signals, following injection of [1-^{13}C]pyruvate, have been detected in Zucker diabetic fatty (ZDF) rats *in vivo*, relative to wild type animals [50]. Increased hepatic glycolysis in the obese animals may explain the greater abundance of lactate in their liver [51-52]. However, both glucokinase and phosphoenolpyruvate carboxykinase, key enzymes that regulate hepatic gluconeogenesis, have been shown to have significantly higher activity in obese Zucker rats, compared to their lean counterparts [53]. As such, these altered hepatic metabolites may reflect both increased gluconeogenesis, as lactate and alanine are used in the liver as precursors for glucose synthesis [54], and glycolysis [55] in the obese animals. Additionally, adipocytes are a significant source of lactate release [56], and therefore the higher abundance of adipose tissue in the obese rats may have contributed to the higher tissue lactate observed.

Higher hepatic glycogen was also seen in the obese rats; there is conflicting evidence regarding glycogen metabolism in the Zucker rat, with findings of both higher [44, 47, 57-58] or similar [51, 59] glycogen content in obese Zucker rat livers *in vivo* and *in vitro*, compared to controls. Similarly, findings regarding the rates of hepatic glycogen synthesis in the Zucker rat are somewhat contradictory, with higher rates in hepatocytes from fasted obese, compared with lean Zucker rats demonstrated [51], yet evidence of a reduced postprandial rate of glycogen synthesis has been seen in obese rats *in vivo*, compared to controls [60], and in hepatocytes [61].

Significantly higher quantities of circulating glycerophosphocholine and hepatic phosphocholine were observed in the obese Zucker animals compared to the lean animals with significantly lower hepatic choline also seen in these animals. Both are important intermediates in the synthesis and metabolism of phosphatidylcholine, an essential component in hepatic VLDL secretion and lipid metabolism [62-64]. Moreover, the plasma obtained from obese rats was found to contain significantly higher amounts of VLDL, as well as LDL, glycerol, and various lipid species. This is indicative of increased lipolysis and hepatic overproduction of lipoproteins in these animals [65-69] and is consistent with previous studies [12, 47, 70-72]. The combination of hyperphagia [25-28] and significantly altered energy metabolism and usage in the obese Zucker rat is thought to contribute to many of the hallmark characteristics of this animal model: hepatic triglyceride accumulation, significantly elevated concentrations of plasma triglyceride [26, 28, 47, 72-73], and elevated secretion of very low-density lipoprotein (VLDL) [67].

Decreased betaine observed in the obese liver, kidney and pancreas, and a lower urinary excretion of methylamine, may be a consequence of this increased demand for VLDL-mediated hepatic triglyceride export. Lipoproteins require a phospholipid outer layer; in rat plasma the most significant phospholipid is phosphatidylcholine [74]. Phosphatidylcholine can be synthesized from phosphatidylethanolamine, or via the 'Kennedy' pathway, from choline. The latter route accounts for approximately 70% of hepatic phosphatidylcholine synthesis [75]. Betaine is the product of choline oxidation and plays an important role in choline metabolism as a methyl donor in the conversion of homocysteine to methionine, producing dimethylglycine *via* the enzyme betaine-homocysteine-methyltransferase (BHMT). This reaction takes place primarily in the liver and kidneys, and secondarily in the pancreas [76-79] (see Figure 4). Hence, the increased demand for VLDL to transport excess triglycerides out of the liver increases the consumption of choline to synthesize phosphatidylcholine, with downstream consequences for betaine abundance. Similarly, reduced urinary methylamine in the obese animals, consistent with previous studies in *ob/ob* [24] and *db/db* [80] mice, may reflect a reduced availability of choline for catabolism either endogenously [81] or *via* gut microbial metabolism [82].

Phenotypic differences were observed in urinary 3-indoxyl sulfate, hippurate and PAG, with all metabolites significantly lower in the urine of the obese animals, relative to the lean. These metabolites clustered together in the HCA, indicating close correlation. As these are microbial-derived products [83-89], these differences imply dysregulation in the activity of the gut microbiota.

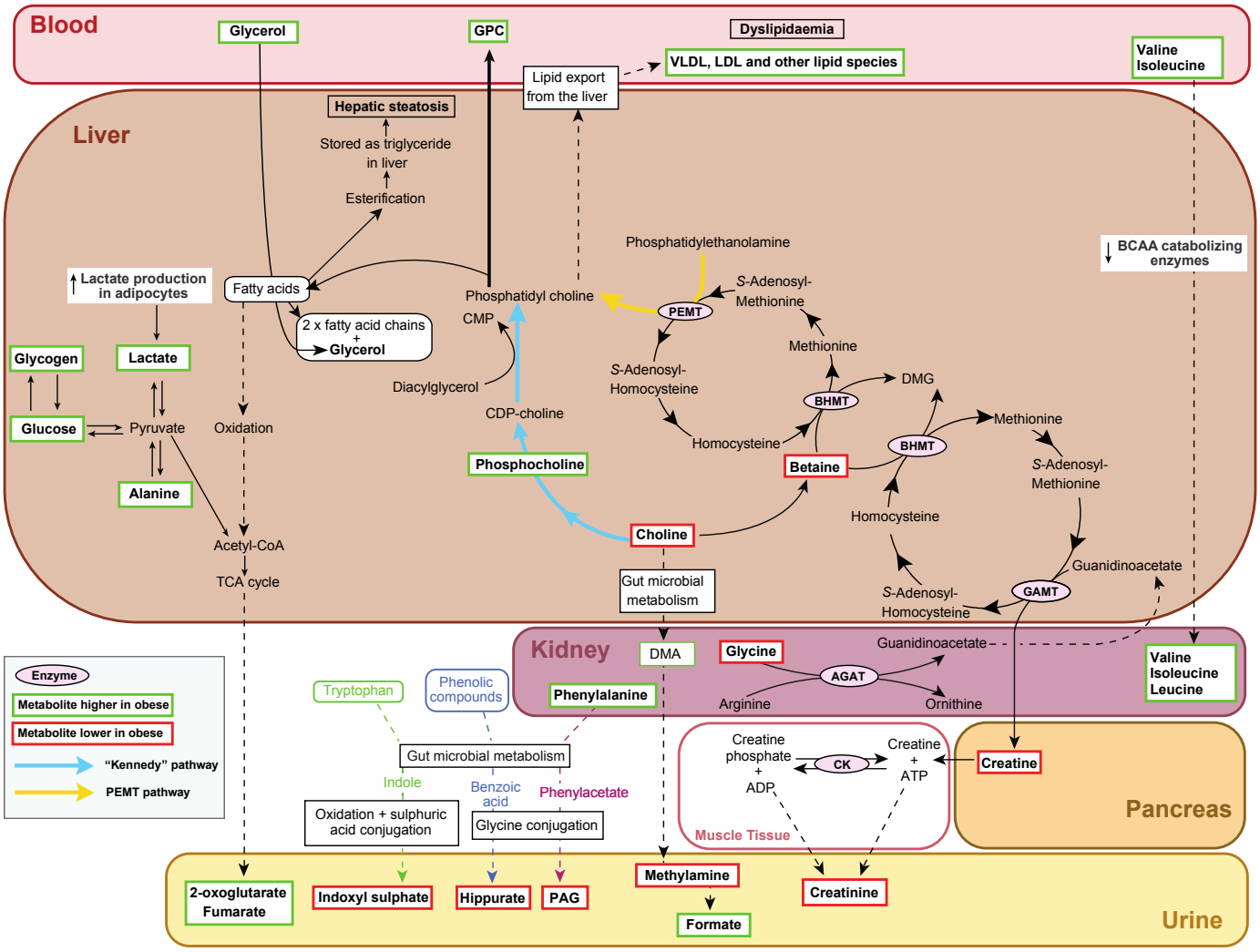

Figure 4: Summary of key metabolic markers characterising the obese Zucker rat in all biological matrices analyzed. Red boxes indicate higher relative concentrations; green indicates lower relative concentrations, observed in the obese, compared to lean rats. ADP, adenosine diphosphate; AGAT, arginine:glycine amidinotransferase; ATP, adenosine triphosphate; BCAA, branched chain amino acid; BHMT, betaine homocysteine methyltransferase; CDP, cytidine diphosphate; CK, creatine kinase; CMP, cytidine monophosphate; CoA, coenzyme A; DMA, dimethylamine; DMG, dimethylglycine; GAMT, guanidinoacetate N-methyltransferase; GPC, glycerophosphocholine; LDL, low-density lipoprotein; PAG, phenylacetylglycine; PEMT, phosphatidylethanolamine N-methyltransferase; TCA, tricarboxylic acid; VLDL, very low-density lipoprotein.

Previous studies of Zucker rats have found results consistent with our findings regarding excretion of hippurate [12] and indoxyl sulfate and PAG [90]. Additionally, hippurate has been shown to be lower in urine from obese individuals [91], and higher in diet-restricted dogs [92]. Furthermore, an inverse relationship between BMI and urinary

phenylacetylglutamine and hippurate has been demonstrated [93], and variation in baseline urinary hippurate has been shown to be predictive of obesity risk [94].

Diet has been shown to alter the microbial metabolism of plant phenolic precursors, resulting in changes in the concentration of hippurate excreted [95]. However, as the composition of the diet was kept the same for all animals throughout the current study, it seems most probable that the phenotype-related variation in hippurate, PAG and indoxyl sulphate observed, reflects variation in the composition or functional activities of the intestinal microbiota. However, our previous analysis of the fecal microbial composition of these animals using 16S rRNA sequencing found no significant phenotypic variance in the relative abundances of phyla or families of bacteria [10]. Together, these data suggest a difference in the biochemical output of the microbiota in these obese animals compared to their lean counterparts, despite no observable differences in the composition of their fecal microbiota at the genus level.

Elevated concentrations of BCAAs (leucine, isoleucine and valine) were seen in the plasma, kidney and pancreatic extracts of obese animals, compared to their lean equivalents, and were found to cluster according to tissue in the HCA performed, indicating close correlation. Additionally, 3-hydroxyisobutyrate (3-HIB), a catabolic intermediate of valine, was found to be higher in the plasma of the obese, compared to lean rats.

Higher blood concentrations of BCAAs have long been associated with the progression of obesity [96-102]. Additionally, the strong association between increased blood BCAA concentrations and the development of insulin resistance has been

confirmed by multiple studies [103-113]. Rats fed a high fat diet supplemented with BCAAs have been shown to develop insulin resistance to the same degree as rats fed a HF diet alone, despite reduced food intake and weight gain [103]. Further to this, obese Zucker rats fed an isonitrogenous diet in which BCAA content was lowered by 45%, showed improved whole-animal insulin sensitivity, muscle glucose uptake and glycogen synthesis. Additionally, the rats on this low-BCAA diet had a lower respiratory exchange ratio, consistent with increased reliance on fatty acid oxidation [114]. Interactions between adipose tissue, BCAA metabolism, and glucose regulation have been proposed [115]. Adipose tissue has been shown to modulate circulating BCCA levels [116], and alterations in BCAA catabolizing enzymes have been observed in the liver and adipose tissue of rodent models of obesity, and also in the adipose tissue of morbidly obese subjects, following bariatric surgery and associated weight loss [102]. Therefore, the altered tissue and blood concentrations of BCAA observed in the obese Zucker rats here may reflect the influence of altered liver and adipose-tissue-derived catabolism of BCAAs in these animals.

In addition to these findings regarding BCAAs, elevated plasma 3-HIB has been observed previously in subjects with type I diabetes [117] and *db/db* mice [118], compared to controls. More recently, investigators found significantly increased 3-HIB in the skeletal muscle of *db/db* mice and in muscle biopsies from people with diabetes. The authors showed that mice administered 3-HIB, accumulated triglycerides and diglycerides in their skeletal muscle and also developed systemic intolerance to a glucose load and insulin resistance, suggesting that 3-HIB acts as a paracrine regulator of trans-endothelial fatty acid flux, linking dysregulated BCAA metabolism with accumulation of lipids in skeletal muscle [119].

Phenylalanine was observed to be present in relatively higher concentrations in extracts of the obese kidney and pancreas, whilst pancreatic tyrosine was observed to be lower in obese pancreatic tissue, relative to the lean animals. As with BCAAs, elevated serum phenylalanine has been associated with obesity [120] and insulin regulation [99, 121]. Phenylalanine metabolism is largely associated with the liver, but minor activity of phenylalanine hydroxylase (PAH), which catalyses the conversion of phenylalanine to tyrosine, has been shown in rat kidney [122], and there is also evidence for the contribution of the pancreas in phenylalanine metabolism [123-124]. Thus, the results here could reflect a reduction in pancreatic PAH activity, leading to accumulation of phenylalanine and reduced tyrosine concentrations in the obese pancreas.

The obese Zucker rats were found to have lower hepatic and pancreatic creatine and urinary creatinine, with glycine also found to be significantly lower in the kidney tissue of the obese rats, compared to the lean animals. A generalized decline in hepatic function has been previously hypothesized to underlie the reduced amount of creatine in the obese Zucker rat liver [46]. Altered choline and SAM metabolism in the obese Zucker rat, as already discussed, may have also contributed to the phenotypic variation in tissue creatine concentrations. The enzyme guanidinoacetate methyltransferase (GAMT) requires SAM in order to methylate guanidinoacetate to produce creatine and S-adenosylhomocysteine (SAH) in the liver and pancreas [125], and thus disturbances in SAM pathways in the obese Zucker rat may have contributed to the phenotypic differences in creatine observed.

Creatine is primarily used as an energy source in muscle. The lower creatine and glycine content of the obese tissues may simply reflect the reduced muscle mass of the obese animals compared to the lean animals, due to reduced physical activity [126]. Creatine and creatine phosphate function as an ATP/ADP ratio buffer in tissues with high and variable energy usage (e.g. skeletal muscle) *via* the enzyme creatine kinase [127]. Elevated urinary creatinine observed in the lean animal samples, compared to the obese, is further evidence of the increased muscle tissue in the lean animals due to spontaneous conversion of creatine and creatine phosphate to creatinine [125, 128-129], with urinary creatinine having been previously positively correlated with lean body mass[130].

Several metabolites were found to have a significant cage-associated variation. This was most pronounced in the urinary metabolites related to gut microbial-host co-metabolism, with trends in other metabolites in liver, kidney and pancreas tissue also evident. This study was designed to attenuate the potential influence of cage environment on host metabolism and fecal bacteria profiles to illuminate the metabolic variation associated with genotype. This was based on the previous results of Waldram *et al.* where each genotype was housed in isolation and clear metabolic and microbial differences were observed between the groups [12].

The trend in gut microbial-associated metabolites is consistent with our previous analysis of the fecal microbiomes of these rats, which found that cage environment had a significant influence on the composition of the fecal microbiota [10]. Here, we found that the excretion of hippurate and 3-HPPA was inversely related to each other

and that the preference of excretion for either metabolite was consistent within each cage.

The cage environment has proved influential during recolonization, following cessation of antibiotic treatment, with a cage-dependent effect observed in both fluorescence *in situ* hybridization analysis of the microbiota, and ^{1}H NMR analysis of urine metabolite profiles [11]. However, the present study differs significantly from this example, in that the animals were already 'colonized' when they were selectively housed together at five weeks of age. Additionally, the obese and lean animals from within the same cage shared the same mother. After birth, the intestine is initially colonized by microbial sources such as the birth canal and faecal material [131-132], and this, together with the initial housing microenvironment, coprophagic behaviour of the animals [133], host genotype, and diet, will have impacted the development of the intestinal microbiota of the animals, as demonstrated by previous investigators [9]. The trends observed in variation among both urinary and tissue metabolites, due to cage environment, underscore the potential impact of cage environment on the metabolism of the host and microbiota, and emphasize the need for control or evaluation of this variable when interpreting results from metabonomic studies.

Conclusions

These results clearly demonstrate the significant impact that the obese phenotype has on all the biological matrices analyzed, reflecting the tissue-specific and systemic impact of obesity. This includes a broad disruption to amino acid, glucose and energy metabolism, which may contribute to the onset of insulin resistance, dysregulation of

lipid metabolism and transport as well as alterations to choline metabolism and host exposure to gut microbial products. This study emphasises the value in metabolic fingerprinting of multiple biological matrices in concert, in order to illuminate the origins of the altered metabolism captured by biofluids. In addition, these results clearly demonstrate and further reinforce the conclusion that the cage environment must be considered as an influential variable in metabonomic studies, especially in the context of host-gut microbial co-metabolites.

Supporting Information:

The following supporting information is available free of charge at ACS website http://pubs.acs.org

Figure S1: Body weights for each strain at each week including pre-study (at four weeks of age); Figure S2: OPLS-DA coefficient loadings plot comparing urine from homozygous lean and obese Zucker rats, including the outlier obese animal from cage 1; Figure S3: OPLS-DA coefficient loadings plot comparing urine from heterozygous lean and obese Zucker rats, including the outlier obese animal from cage 1; Figure S4: OPLS-DA coefficient loadings plot comparing urine from heterozygous lean and obese Zucker rats, excluding the outlier obese animal from cage 1; Figure S5: OPLS-DA coefficient loadings plot comparing plasma from heterozygous lean and obese Zucker rats, excluding the outlier obese animal from cage 1; Figure S6: OPLS-DA coefficient loadings plot comparing liver samples from heterozygous lean and obese Zucker rats; Figure S7: OPLS-DA coefficient loadings plot comparing kidney samples from heterozygous lean and obese Zucker rats.

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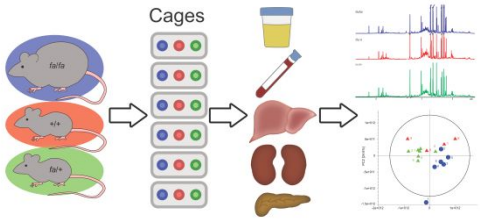
Cages