Systemic Characterization of an Obese Phenotype in the Zucker Rat Model Defining Metabolic Axes of Energy Metabolism and Host-Microbial Interactions

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ABSTRACT: The Zucker (*fa*/*fa*) rat is a valuable and extensively utilized model for obesity research. However, the metabolic networks underlying the systemic response in the obese Zucker rats remain to be elucidated. This information is important to further our understanding of the circulation of the microbial or host-microbial metabolites and their impact on host metabolism. ^{1}H Nuclear Magnetic Resonance spectroscopy-based metabolic profiling was used to probe global metabolic differences in portal vein and peripheral blood plasma, urine and fecal water between obese (*fa*/*fa, n*=12) and lean (*fa*/+, *n*=12) Zucker rats. Urinary concentrations of host-microbial co-metabolites were found to be significantly higher in lean Zucker rats. Higher concentrations of fecal lactate, short chain fatty acids (SCFAs), 3-hydroxyphenyl propionic acid and glycerol, and lower levels of valine and glycine were observed in obese rats compared with lean animals. Regardless of phenotype, concentrations of SCFAs, tricarboxylic acid cycle intermediates, and choline metabolites were higher in portal vein blood compared to peripheral blood. However, higher levels of succinate, phenylalanine and tyrosine were observed in portal vein blood compared with peripheral blood from lean rats but not in obese rats. Our findings indicate that the absorption of propionate and acetate, choline and TMA are independent of the Zucker rat phenotypes. However, urinary host-microbial co-metabolites were highly associated with phenotypes, suggesting distinct gut microbial metabolic activities in lean and obese Zucker rats. This work advances our understanding of metabolic processes associated with obesity, particularly the metabolic functionality of the gut microbiota in the context of obesity.

KEYWORDS: Metabonomics·Metabolomics·Zucker rats· Obesity· Metabolic Profiling· NMR spectroscopy

■ INTRODUCTION

Obesity and its co-morbidities have become a severe health and socioeconomic problem[1]. Although numerous obesity studies have been performed in humans, animal models remain valuable for providing complementary insights into disease mechanisms[2]. The Zucker (*fa*/*fa*) rat has been one of the most commonly used rat models to study obesity over the last three decades[2]. Obesity in these rats is caused by a mutation in a single recessive gene (*fa*) of the leptin receptor and is characterized by hyperphagia, hyperlipidemia, hypercholesterolemia, leptin resistance, hyperinsulinemia, and development of adipocyte hypertrophy and hyperplasia[3].

Metabolic profiling is a rapidly developing systems biology approach and powerful method for studying metabolic alterations in a biological system using both global and targeted approaches for characterizing the biochemical composition of complex biological matrices such as plasma, urine, fecal water and tissue extracts[4]. ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy is a robust and reproducible platform commonly used for metabolic profiling; it is non-invasive and requires minimal sample preparation[4-5]. A ^{1}H NMR spectroscopy-based metabonomics study by Waldram *et al.*[6] has reported higher levels of plasma lipids, choline, and branched chain amino acids (BCAAs) and higher urinary concentrations of propionate, acetate and 3-methylglutarate in Zucker obese (*fa*/*fa*) compared with the Zucker lean (*fa*/+) or (+/+) phenotype. Although many of the metabolic features that characterize the obese Zucker rat relate to energy metabolism, a core component of the phenotype suggests a differential signature in the obese phenotype arising from the gut microbiota. The role of the gut microbiota in creating an obesogenic environment is supported by many studies[7]. However, another previous

analysis of the fecal microbial composition of lean and obese Zucker rats carried out by Lees *et al.*[8] found no significant phenotypic difference, with variation attributed to age and animal husbandry. Given the disagreement in findings between Waldram and Lees' studies with regards to microbial composition of lean and obese Zucker rats, it is worth noting the different animal housing strategies employed: animals housed according to a single or mixed strains. In our study, we address this issue by housing animals individually. These previous studies suggest that a shift from investigation of biochemical composition to the metabolic functionality of the gut microbiota in obesity could be of great benefit.

Thus far, the consistency of the metabolic changes in Zucker rats over time and the contribution of gut microbial metabolites, delivered *via* the portal vein, to host metabolism and phenotype remains poorly understood. In this study, we used ^{1}H NMR spectroscopy in combination with multivariate statistical analyses, to monitor dynamic changes in urinary and fecal water profiles obtained from obese and lean Zucker rats over 4 weeks. Furthermore, the biochemical composition of the portal vein and peripheral blood plasma were characterized, for the first time in Zucker obese animals, to investigate the uptake of the gut microbial metabolites and their systemic circulation and metabolism in the host.

■ EXPERIMENTAL SECTION

Animal Model and Sampling.

Twelve male Zucker obese (*fa*/*fa*) and 12 lean control (*fa*/+) rats were included in the study and individually caged. Urinary and fecal samples were collected weekly up to 4 weeks and directly stored at -80 °C. At week 4, the peripheral and portal vein blood were collected to obtain plasma, which was snap-frozen and stored at -80 °C. Detailed description of the animal experiment, oral glucose tolerance test (OGTT) and the measurement of fasting insulin levels were described in Supporting Information (SI).

^{1}H NMR Spectroscopic Analyses of Biofluids and Data Analysis.

Plasma, urine and fecal water (see SI for fecal water extraction) samples were analyzed using a 600 MHz spectrometer (Bruker Avance III, Bruker Biospin, Germany) according to previously published protocols[4,5]. The resulting spectral data of all biofluids were pre-processed and analyzed using multivariate statistical analyses including principal component analysis (PCA) and orthogonal signal correction-projection to latent structures-discriminant analysis (O-PLS-DA). The metabolite profiles of all biofluids were correlated with body weight and fasting insulin levels, as described in SI.

■ RESULTS

Body Weight, Food Intake, Oral Glucose Tolerance Test and Fasting Insulin

Obese and lean Zucker rats were found to be different for all the phenotypic traits measured. The mean body weight of obese rats (*fa*/*fa*) was significantly higher than that of

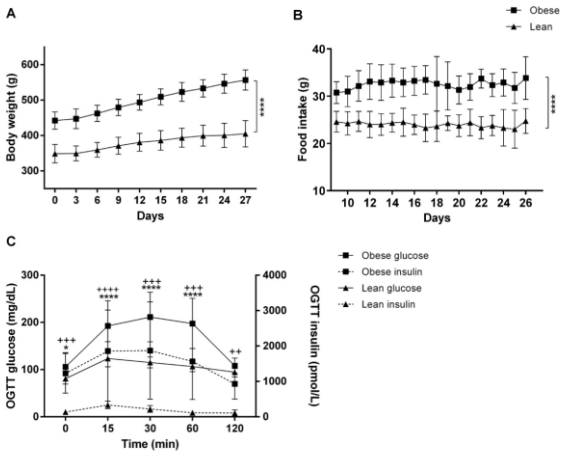

Figure 1. (A) Measurement of body weight; (B) food intake, data are shown as mean ± SD (****p < 0.0001) and statistical differences determined by two-way ANOVA and (C) oral glucose tolerance test (OGTT) of lean and obese Zucker rats (n=12 per group). OGTT glucose (solid line) and insulin (dashed line) measurements of Zucker rats. Data are shown as mean ± SD (*p < 0.05; ****p < 0.0001 of OGTT glucose and ++p < 0.01; +++p < 0.001; ++++p < 0.0001 of OGTT insulin by two-way ANOVA).

lean rats (obese: 499.5 ± 40.9 g *vs.* lean: 379.5 ± 21.2 g, $p < 0.0001$). Obese rats had higher food intake compared with lean animals over the 4-week study period (obese: 32.5 ± 0.9 g *vs.* lean: 24 ± 0.5 g, $p < 0.0001$, using two-way ANOVA) (Figures 1A and 1B). Fasting glucose levels of obese and lean Zucker rats were significantly different (obese: 105.8 ± 8.7 mg/dL *vs* lean: 80.8 ± 3.9 mg/dL, $p = 0.0033$).

Similarly, both glucose and insulin levels were significantly higher in obese animals compared with lean rats during the OGTT (at 15, 30 and 60 mins). At 120 min, the glucose level normalized in obese rats and was comparable with lean rats but the insulin levels remained higher. The glucose level of obese rats persisted at a high concentration for 1 hour after feeding, whilst in lean rats it started to decrease at 15 min (Figure 1C). Obese Zucker rats showed significantly higher fasting insulin levels (1334.0 ± 134.2 pmol/L) than lean rats (135.7 ± 17.6 pmol/L, $p < 0.001$) (Figure 1C).

Urinary Metabolic Profiles of Obese (*fa*/*fa*) and Lean (*fa*/+) Zucker Rats

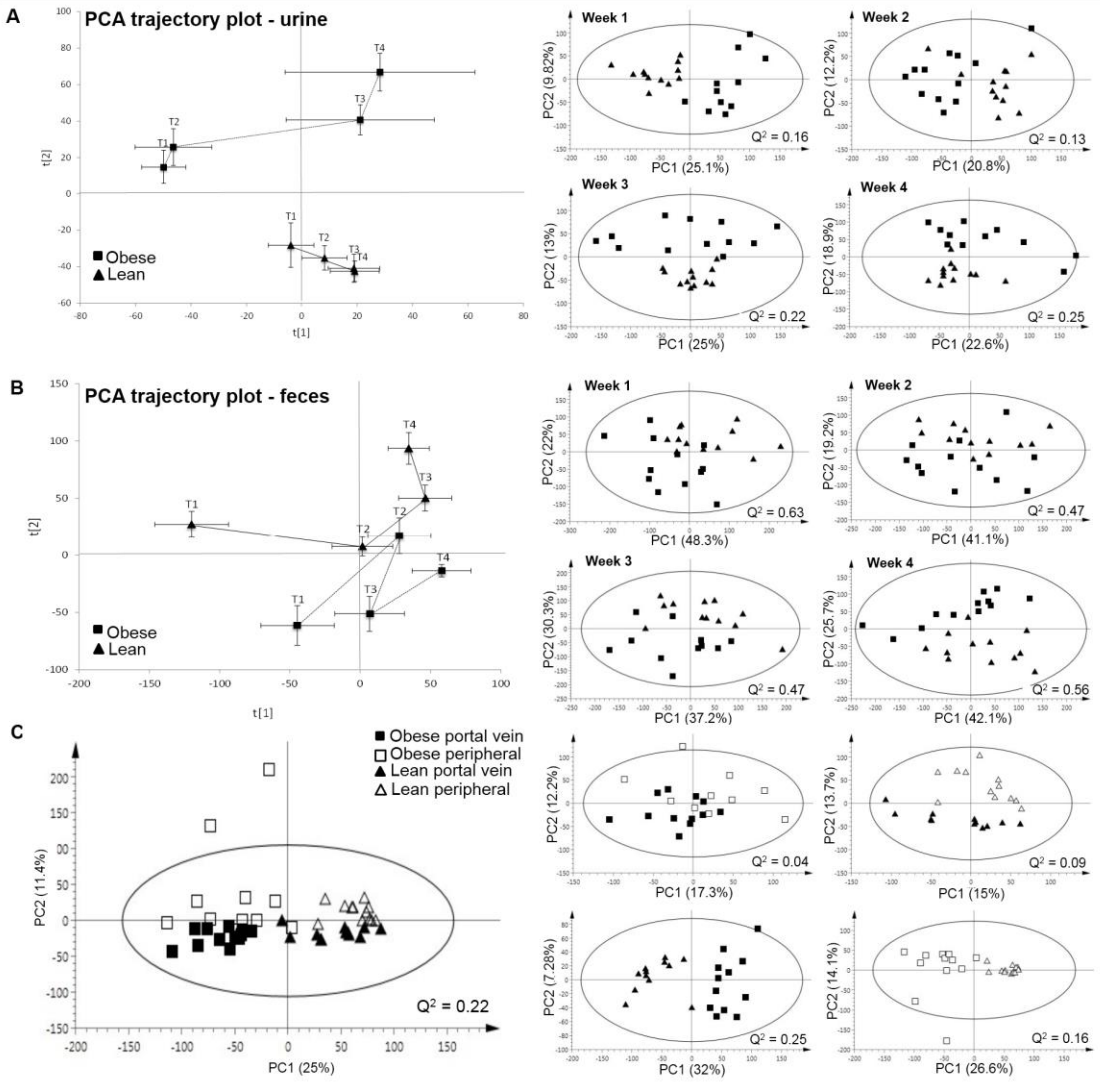

Figure 2. Principal component analysis of urinary (A), fecal (B) metabolic profiles of obese and lean Zucker rats over 4-week period and of obese peripheral, lean peripheral, obese portal vein and lean portal vein blood plasma (C) metabolic profiles at week 4. PCA trajectory scores plot of urinary and fecal data sets obtained from the average scores of PC1 against PC2 of each strain of rat at a particular time point and error bars are expressed by the standard error of mean of PC1 and PC2 of each time point. Percentage on each PC represents variation explained by each principal component and Q^2 represents predictive ability of the model.

A shift in global urinary metabolite profiles of both lean and obese rats was observed over a 4-week period, manifesting in the PCA trajectory scores plot as a time-related shift along the first principal component (PC1) and a separation of obese rats from lean rats along the second principal component (PC2) (Figure 2A). PCA scores plots based on each discrete time point showed clear clustering of the two groups of rats along PC1 or PC2 (Figure 2A) indicating that the two strains could be differentiated from the first time point and that this metabolic difference persisted for the duration of the study. O-PLS-DA analysis was applied to investigate the metabolites that were discriminant between the obese and lean classes at each time point (Table 1). The loadings of the pairwise O-PLS-DA models identified significantly higher urinary levels of 2-hydroxyvalerate, 2-hydroxybutyrate, 2-ketoisocaproate, methylmalonate, *N*-epsilon-acetyllysine, *N*-acetylcysteine, 4-cresyl glucuronide, creatinine, proline, allantoin, indoxyl sulfate (IS) and unknown 3 (δ^1H 8.08 (s); δ^1H 8.57 (s)) in the lean Zucker rats, whereas 1-methylnicotinamide, unknown 1 (δ^1H 0.91 (s); δ^1H 1.73 (t)) and unknown 4 (δ^1H 8.32 (d, J=2.65)) levels were found in higher concentrations in obese rats. The relative concentrations of 2-hydroxybutyrate, methylmalonate, creatinine, proline and phenylacetylglycine (PAG) were consistently lower in obese rats across the four time points, whilst other metabolites such as 2-hydroxyvalerate, 2-ketoisocaproate were discriminating features at two or three time points. The O-PLS regression

Table 1. Summary of metabolic changes in urinary (u) and fecal (f) ^{1}H NMR profiles

Metabolites	Chemical shift	O-PLS-DA models of obese and lean Zucker rats								O-PLS regression
		Week 1 (u):R^2X=32.9%, Q^2Y=0.92, p = 0.001 (f):R^2X=69.2%, Q^2Y=0.48, p = 0.001		Week 2 (u):R^2X=30%, Q^2Y=0.59, p = 0.001 (f):R^2X=54.8%, Q^2Y=0.38, p = 0.005		Week 3 (u):R^2X=30.3%,Q^2Y=0.65, p = 0.001 (f):R^2X=60%, Q^2Y=0.39, p = 0.018		Week 4 (u):R^2X=33.5%,Q^2Y=0.85, p = 0.001 (f):R^2X=66%, Q^2Y=0.71, p = 0.001		Body weight[b] (u):R^2X=24.8%,Q^2Y=0.66, p = 0.001 (f):R^2X=75.3%, Q^2Y=0.57, p = 0.001
2-hydroxyvalerate	0.84 (t)	-0.9057 (u)	****	-0.7163 (u)	***					
2-hydroxybutyrate	0.86 (t); 1.60 (m); 1.71 (m)	-0.9201 (u)	****	-0.7458 (u)	***	-0.6820 (u)	**	-0.7903 (u)	***	-0.6461 (u)
Butyrate	0.90 (t); 1.56 (m); 2.16 (t)	+0.8140 (f)	****	+0.6433 (f)	**			+0.6218 (f)	**	
Unknown1	0.91 (s); 1.73 (t)	+0.8330 (u)	***	+0.8093 (u)	***	+0.5626 (u)	**	+0.9016 (u)	****	+0.6234 (u)
2-ketoisocaproate	0.94 (d); 2.08 (m); 2.60 (d)	-0.7609 (u)	**			-0.7675 (u)		-0.7728 (u)		-0.6544 (u);
Leucine	0.96 (t); 1.71 (m)									-0.4898 (f)
Isoleucine	1.00 (d); 0.92 (t)									-0.5066 (f)
Valine	0.99 (d); 1.02 (d)					-0.6779 (f)				-0.5208 (f)
Propionate	1.06 (t); 2.19 (q)							+0.8248 (f)	****	+0.5425 (f)
Methylmalonate	1.26 (d)	-0.7644 (u)	****	-0.633 (u)	**	-0.7353 (u)	***	-0.8301 (u)	**	-0.6991 (u)
Lactate	1.33 (d); 4.11 (q)					+0.7036 (f)	**	+0.6808 (f)	***	
Acetate	1.92 (s)					+0.6865 (f)	**	+0.7308 (f)	****	
N-epsilon-acetyllysine	1.99 (s)		***	-0.6556 (u)	*			-0.7728 (u)	****	-0.6013 (u)
N-acetylcysteine[a]	2.05 (s)	-0.8774 (u)		-0.7045 (u)				-0.9080 (u)	***	-0.786 (u)
N-acetylglucosamine	2.06 (s); 5.21 (d)	-0.7761 (f)	**							
4-cresyl glucuronide	2.30 (s); 7.05(d); 7.2 (d)	-0.7002 (u)	***	-0.7132 (u)	**			-0.7848 (u)	****	-0.537 (u)
3-hydroxyphenyl propionic acid	2.47 (t); 2.84 (t); 7.25 (t); 6.76 (dd); 6.8 (s); 6.88 (d)							+0.7289 (f)	****	
Creatinine	3.04 (s); 4.05 (s)	-0.9108 (u)	****	-0.7776 (u)	****	-0.7739 (u)	****	-0.9007 (u)	****	-0.6284 (u)
Glycine	3.57 (s)	-0.6152 (f)						-0.6299 (f)		
Glycerol	3.58 (dd); 3.65 (dd)	0.6814 (f)				+0.6814 (f)	**	+0.7366 (f)	***	
Proline	4.14(d); 2.33 (m); 2.02 (m); 3.32 (m); 3.42 (m)	-0.8475 (u)	*	-0.8074 (u)	*	-0.6930 (u)	**	-0.7479 (u)	**	
1-methylnicotinamide	4.48 (s); 8.19 (t); 8.90 (d); 8.96 (d); 9.28 (s)	+0.8902 (u)	****	+0.8106 (u)	****			+0.7993 (u)	****	+0.5188 (u)
Allantoin	5.40 (s)					-0.7194 (u)		-0.8457 (u)		-0.6842 (u)
Unknown2	5.64 (d); 8.57 (s)	-0.7663 (f)	**							-0.5252 (f)
Cytosine	5.97 (d); 7.51 (d)							+0.8190 (f)	****	+0.5498 (f)
Phenylacetylglycine	7.42 (m); 7.35 (m); 3.66 (s); 3.74 (d)	-0.6697 (u)	**	-0.6723 (u)	**	-0.5228 (u)	*	-0.7809 (u)	****	-0.5421 (u)
Indoxyl sulfate	7.36 (s); 7.50 (d); 7.69 (d); 7.21 (t); 7.27 (t)	-0.8275 (u)	***	-0.7128 (u)	*			-0.7515 (u)	****	-0.6746 (u)
Unknown3	8.08 (s); 8.57 (s)							-0.7812 (u)	***	
Unknown4	8.32 (d, *J*=2.65 Hz)	+0.8806 (u)	****	+0.7187 (u)	*	0.6871 (u)	**	+0.8683 (u)	****	+0.592 (u)

"+" indicates higher correlation in obese Zucker rats, whereas "–" indicates higher correlation in lean Zucker rats. Abbreviations: s, singlet; d, doublet; dd, double of doublets; t, triplet; bs, broad singlet; m, multiplet; q, quartet. [a]Database in Chenomx. [b]"+" indicates higher correlation of metabolites with increasing body weight, whereas "– "indicates higher correlation with decreasing body weight. *, **, *** and **** indicate significant levels of metabolite differences between lean and obese Zucker rats of each time point at p<0.05, p<0.01, p<0.001 and p<0.0001, respectively using two-tailed heteroscedastic t-test with Benjamini-Hochberg correction. P values of all models were derived from permutation tests (n=1000).

Table 2. Summary of metabolic changes in plasma ^{1}H NMR profiles

Metabolites	Chemical shift	O-PLS-DA models								O-PLS regression models	
		obese peripheral (+)[a] vs. obese portal vein (-) (R^2X=25.7%, Q^2Y=0.71, p = 0.001)		lean peripheral (+) vs. lean portal vein (-) (R^2X=19.9%, Q^2Y=0.79, p = 0.001)		lean peripheral (+) vs. obese peripheral (-) (R^2X=37.8%, Q^2Y=0.88, p = 0.001)		lean portal vein (+) vs. obese portal vein (-) (R^2X=32%, Q^2Y=0.83, p = 0.001)		Peripheral plasma with Body weight[b] (R^2X=37.4%, Q^2Y=0.73, p = 0.001)	Peripheral plasma with Fasting insulin[c] (R^2X=37.2%, Q^2Y=0.63, p = 0.001)
Valine	0.99 (d); 1.02 (d)					-0.6779	*				
Isoleucine	1.00 (d); 0.92 (t)					-0.6462	*	-0.8221			
Propionate	1.06 (t); 2.19 (q)	-0.7762	***	-0.6460	****						
D-3-hydroxybutyrate	1.19 (d); 2.31 (dd); 2.41 (dd)			+0.8581	**						
Lactate	1.33 (d); 4.11 (q)					-0.7229	***				
Alanine	1.48 (d)					+0.8294				-0.5752	
Acetate	1.92 (s)	-0.7003	**	-0.7786	***						
Glutamine	2.14 (m); 2.46 (m)					+0.8759	****	+0.6807	****	-0.7962	-0.8046
Proline	4.14(d); 2.33 (m); 2.02 (m); 3.32	-0.9053		-0.8688							
Pyruvate	2.38 (s)	-0.8901	**	-0.8122	***						
Succinate	2.41 (s)			-0.8920	****			+0.8098	****		-0.5918
Trimethylamine	2.89 (s)	-0.8850	****	-0.7965	****						
Creatine	3.04 (s); 3.93 (s)					+0.6881	***	+0.7818	****		
Malonate	3.12 (s)	-0.7522	****	-0.9043	****	+0.7442	***	+0.7346	**	-0.713	
Phenylalanine	3.13 (dd); 3.28 (dd); 3.39 (m); 4.00 (dd); 7.39 (m)			-0.8606	****						-0.6763
choline	3.17 (s)	-0.8497	****	-0.8878	***					-0.7368	
2-deoxycytidine	6.03 (d); 6.25 (t); 7.83 (d)					+0.7613	****				
Fumarate	6.53 (s)	-0.8308	*	-0.7770	****						
Tyrosine	3.06 (dd); 3.26 (dd); 3.94 (dd); 6.90 (d); 7.19 (d)			-0.6701	*	+0.7676	****	+0.8388	****	-0.7134	-0.6126
Lipids CH_3-	0.84 (bs)					-0.8515	****	-0.9285	****	+0.8287	+0.749
Lipids $(CH_2)_n$	1.25 (bs)					-0.9319	****	-0.9032	**	+0.8067	+0.7879
Lipids CH_2C=C	2.00 (bs)					-0.7925	***	-0.6962	***	+0.6251	+0.8243
Lipids =C-CH_2-C=	2.76 (bs)					-0.8531	****	-0.9402	****	+0.7805	+0.766
Lipids CH=CH	5.29 (bs)					-0.847	****	-0.914	****		+0.8102

"+" or "−" indicates higher correlation in either group of O-PLS-DA pairwise comparison models. Abbreviations: s, singlet; d, doublet; dd, double of doublets; t, triplet; bs, broad singlet; m, multiplet; q, quatet. [b,c]"+" indicates higher correlation of metabolites with increasing body weight or fasting insulin whereas "− "indicates higher correlation with decreasing body weight or fasting insulin. *, **, *** and **** indicate significant levels of metabolite differences between lean and obese Zucker rats of each pairwise comparison at p<0.05, p<0.01, p<0.001 and p<0.0001, respectively using two-tailed heteroscedastic t-test with Benjamini-Hochberg correction. P values of all models were derived from permutation tests (n=1000).

model of urinary profiles against insulin levels was not robust (R^2X=7.91%; Q^2Y=0.11, CV-ANOVA p=0.102). However, O-PLS regression analysis (R^2X=24.8%, Q^2Y=0.66, CV-ANOVA p<0.001) of urinary metabolic profiles against body weight showed that 1-methylnicotinamide was positively correlated with body weight, whereas metabolites such as 4-cresyl glucuronide, creatinine, PAG and IS were inversely correlated with body weight (Table 1).

Fecal Water Metabolic Profiles of Obese and Lean Rats

Clustering of fecal water profiles from obese versus lean Zucker rats was observed along the second component in both the time-related PCA trajectory plot and PCA scores plots at each individual time point (Figure 2B). Different from the urinary PCA trajectory, fecal profiles of lean and obese rats exhibited a similar degree of variation in the PCA plot. Higher relative concentrations of lactate, short chain fatty acids (SCFAs including propionate, acetate, and butyrate), cytosine, and 3-hydroxyphenylpropionic acid (3-HPPA) and glycerol, and lower levels of *N*-acetylglucosamine, valine and glycine were observed in obese rats compared with lean animals (Table 1). As with the urinary data, correlation between the fecal metabolites and insulin levels was not found to be significant (O-PLS regression model: R^2X=13.23%, Q^2Y<0, CV-ANOVA p=0.192). However, animal body weight was found to be positively correlated with fecal propionate and cytosine, and inversely correlated with BCAAs (e.g. leucine, isoleucine and valine) and an unknown metabolite 2 (δ^1H 5.64 (d), 8.57 (s)) (Table 1) based on the O-PLS regression analysis.

Metabolic Profiles of Systemic and Portal Vein Blood Plasma from Obese and Lean Zucker Rats

The magnitude of the metabolic differences between obese and lean rats was greater than that observed between portal and peripheral blood with the genotypes separating along the first principal component and the peripheral versus portal vein plasma separating along the PC2 (Figure 2C). Within the lean or obese group, the peripheral and portal vein plasma metabolite profiles separated, with a clearer clustering observed in lean genotype along the second principal component (Figure 2C). A stronger separation between the genotypes in either peripheral or portal vein blood was observed along PC1, with portal vein lean rat plasma samples clustering much tighter. Higher concentrations of SCFAs (e.g. propionate and acetate), metabolites involved in the tricarboxylic acid (TCA) cycle (e.g. pyruvate and fumarate), proline, choline, trimethylamine (TMA) and malonate were found in portal vein blood compared with the peripheral blood in both obese and lean animals. Additionally, the portal vein blood of lean rats carried higher levels of succinate, phenylalanine and tyrosine than the peripheral blood but these metabolites were not found to differentiate peripheral and portal blood in obese rats. In contrast, D-3-hydroxybutyrate was found to be higher in peripheral blood plasma compared with portal vein blood in the lean genotype. In both portal and peripheral blood plasma, obese Zucker rats had higher levels of lipids and isoleucine and lower levels of glutamine, creatine, malonate and tyrosine in contrast to the lean animals. In obese animals, circulating blood plasma levels of BCAAs including valine and isoleucine, and lactate were higher, and 2-deoxycytidine was lower, compared to lean animals. Succinate was found to be higher in the portal vein blood of the lean, compared to obese rats (Table 2). In order to probe the association between the plasma metabolic phenotype and the concentration of fasting insulin and body weight, the peripheral plasma metabolite profiles were correlated with body weight and fasting insulin levels, separately, using an O-PLS regression analysis. Both body weight and fasting insulin concentrations were directly correlated with plasma lipid concentrations, whereas glutamine and tyrosine were inversely correlated. Alanine, malonate and choline inversely correlated with body weight, whereas succinate and phenylalanine were inversely correlated with fasting insulin levels.

Inter-Compartmental Correlation Analysis of Plasma, Urinary and Fecal Metabolites

To probe the inter-correlation among metabolites from urine, feces and plasma and uncover the differences in these correlation structures between lean and obese rats,

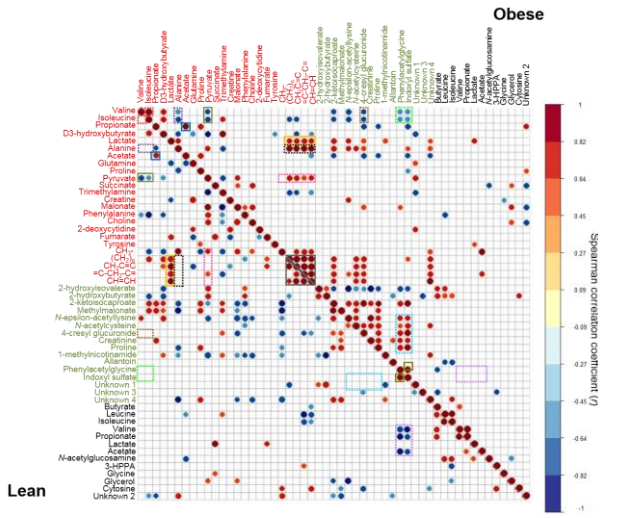

Figure 3. Inter-compartmental correlation map of plasma (red), urinary (green) and fecal (black) metabolites from Zucker obese and lean rats with *p-value* < 0.05. Red denotes positive correlation, blue denotes negative correlation, and blank denotes no significant correlation. Key: 3-HPPA, 3-hydroxyphenyl propionic acid. Dashed-line box indicates different metabolic structures, whereas solid-line box indicates similar metabolic structures between lean and obese rats.

we constructed correlation matrices for lean and obese animals separately without clustering to allow the direct comparison between two genotypes (Figure 3). The most prominent observations were the strong inter-correlations of lipid signals and their significantly positive correlation with glycolysis end products including pyruvate, lactate and alanine, 2-ketoisocaproate derived from leucine metabolism, and 4-cresyl glucuronide in the obese rats, whereas in lean animals, only the correlation between lactate and all lipid signals was observed. In addition, valine and isoleucine were positively correlated in obese rats, which were inversely correlated with alanine, pyruvate, IS and PAG. Positive correlations between plasma acetate and propionate, and between urinary PAG, IS and 4-cresyl glucuronide were observed in both groups. However, these two urinary host-microbial co-metabolites were positively correlated with metabolites such as 4-cresyl glucuronide and creatinine in obese rats, and inversely correlated with fecal valine, propionate and acetate in lean animals.

■ DISCUSSION

Obese (*fa*/*fa*) and lean (*fa*/+) Zucker rats were phenotypically distinct in all biological matrices measured using a ^{1}H NMR spectroscopy-based metabolic profiling approach. We have reported for the first time the global metabolic profiles of portal vein plasma of hyperinsulinemic obese Zucker rats that may provide the baseline metabolic information to further understand this particular type of obese animal model. As expected, obese Zucker rats were significantly hyperphagic compared to lean controls (Figure S1), resulting in a greater food intake and body weight[9].

Higher levels of urinary creatinine and plasma creatine were observed in the lean Zucker rats as compared to their obese counterparts, which is consistent with a previous study in Zucker rats[5]. De Castro and colleagues (2013) also reported the higher level of creatine in the white adipose tissue of lean Zucker rats compared with that of obese Zucker rats at two months old, but such a change was not observed in rats at six months old[10]. Creatinine is derived from creatine phosphate in muscle[11] and formation of creatinine results from the loss of water molecule from creatine. Creatinine is transferred to kidney through the blood and eliminated from the body by glomerular filtration and partial tubular excretion[12]. 24-hour urinary excretion of creatinine has been reported to be significantly correlated with fat-free mass[11]. A cohort of 170 healthy individuals also

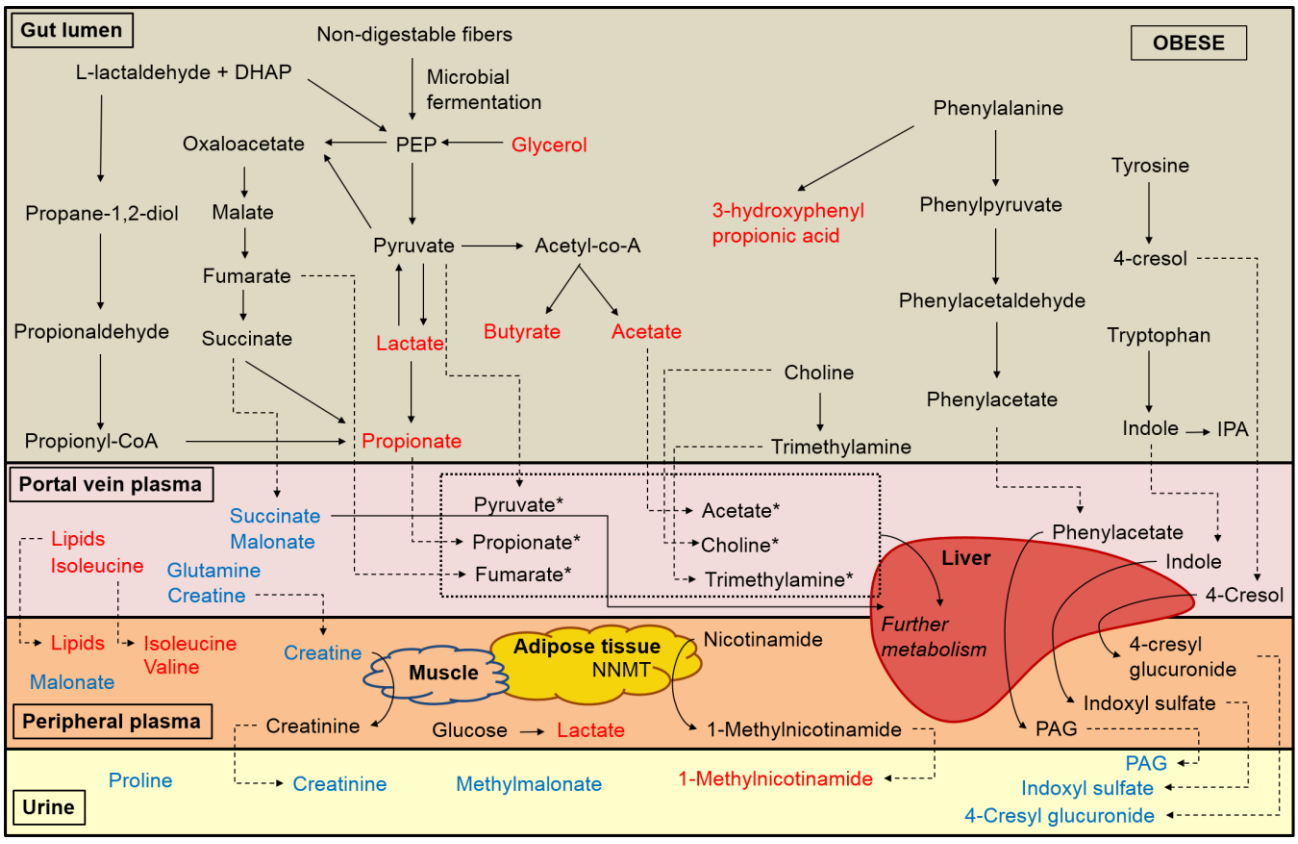

Figure 4. Schematic illustration of systemic changes observed in biofluids from obese Zucker rats. Red indicates higher relative concentration, whereas blue indicates lower relative concentration of metabolites in obese rats compared with lean animals. Key: IPA, indole-3-propionic acid; NNMT: nicotinamide *N*-methyltransferase; PAG, phenylacetylglycine; PEP, phosphoenolpyruvate. * indicates that these metabolites are higher in portal vein blood plasma in contrast to peripheral blood plasma independent of the animal genotypes.

showed that higher muscle mass was associated with higher levels of physical activity, and that serum and urinary creatinine were positively correlated with the muscle mass and body weight with a greater degree of correlation with muscle mass[13]. In the current study, the excretion of lower levels of urinary creatinine in the obese rats may be indicative of lower lean mass, compared with the lean rats.

In the obese Zucker rats, elevated levels of urinary 1-methylnicotinamide were observed, which is in agreement with previous findings in high fat diet (HFD)-induced male C57BL/6N obese mice[14]. 1-methylnicotinamide is a product of nicotinamide catalysis *via* nicotinamide *N*-methyltransferase (NNMT) which can be found in both liver and adipose tissue[15]. It has been found that 3T3-L1 adipocytes and murine adipose tissue express high levels of NNMT activity, resulting in production of 1-methylnicotinamide, which is excreted into urine, and *S*-adenosyl-L-homocysteine, which is further metabolized into homocysteine by *S*-adenosyl-L-homocysteine hydrolase[16]. Although high hepatic NNMT expression was shown to improve lipid parameters through sirtuin 1 stabilization[17], Kraus et al (2014) reported that high adipose NNMT expression correlates with adiposity in both humans and mice[18]. Nicotinamide can be salvaged for the re-synthesis of NAD^+ that is required for the fuel oxidation. When NNMT is highly expressed, nicotinamide may not be salvageable, limiting fuel oxidation and promoting the storage of fat[18]. Hence, the higher level of 1-methylnicotinamide indicates a rich pool of NNMT in the adipose tissue of the obese rats.

Higher concentrations of fecal SCFAs were found in obese Zucker rats compared with lean rats, which could result from the increased bioavailable fermentation substrates from higher food intake and/or increased microbial fermentation of non-digestible carbohydrates by the gut microbiota in the obese rats. Higher cecal concentrations of SCFAs have been reported in genetically obese C57BL/6J (*ob*/*ob*) mice, indicating that the microbiota from obese mice had greater ability to extract energy from food[19]. We also found higher levels of propionate and acetate, but not butyrate, in the portal vein blood compared with peripheral blood in both lean and obese animals. This could be attributed to consumption of a large part of butyrate by colonocytes as an important energy source. In contrast, acetate and propionate are absorbed and further utilized in the liver for synthesis of cholesterol and long-chain fatty acids, and hepatic gluconeogenesis[20]. Concentrations of acetate and propionate in the portal vein blood plasma did not significantly differ between the lean and obese animals given the elevated fecal SCFAs, suggesting that the absorption of acetate and propionate through hepatic portal vein circulation may not directly contribute to the obese phenotype in Zucker rats. Propionate can be produced *via* three known bacterial fermentation pathways (Figure 4) including the succinate pathway, where fumarate and succinate are produced as intermediates; the acrylate pathway, where pyruvate and lactate are intermediates; and the propanediol pathway, where propane-1,2-diol is an intermediate[14]. These intermediates including fumarate and pyruvate were shown to be absorbed into the hepatic portal circulation in both obese and lean animals in our study. Higher levels of hepatic portal succinate in the lean animals were observed in comparison to the obese animals, but its level in the peripheral blood did not differ between the two groups. This finding suggests an elevated uptake of succinate and enhanced hepatic succinate metabolism (e.g. TCA cycle and adenosine triphosphate generation in mitochondria) in the lean animals. In lean Zucker rats, the aforementioned succinate pathway for synthesizing propionate could be suppressed, resulting in increased succinate availability in the gut lumen, leading to its increased portal vein blood concentrations.

The relatively high concentrations of fecal lactate found in the obese samples suggests an imbalance of lactate-producing (e.g. *Bifidobacteria*) and lactate-utilizing bacteria (e.g. *Eubacterium hallii*, *Anaerostipes caccae*) in the gut. A previous study reported the highest abundance of lactate-producing bacteria in T2DM, followed by obese and lean participants[21]. A positive correlation between fecal SCFAs and fecal lactate was observed in the current study. Lactate is an intermediate metabolite of SCFAs present in low concentrations (<5 mmol/L) in fecal samples from healthy individuals, but it may accumulate in concentrations up to 90 mmol/L in patients with ulcerative colitis as previously reported[22], which could be attributed to curtailment of lactate utilization at pH of 5.2.

We also detected higher levels of choline and TMA in the portal vein blood compared with peripheral blood independent of the genotypes of the animals. Choline is a common dietary component and can be metabolized into TMA *via* the gut microbiota[23]. The unmetabolized choline and resulting TMA are absorbed and the latter is known to be further oxidized in the liver to trimethylamine-*N*-oxide involving flavin-containing monooxygenases (FMO)[24]. Although the strain-specific and genetically determined selections of the gut microbiota of high fat diet-fed animals were reported to be associated with choline metabolism[23], TMA and choline did not differ between two genotypes in the current study, indicating that the obesity phenotype of the Zucker rats could be independent of the microbial metabolism of choline.

Microbial metabolism of aromatic amino acids (e.g. phenylalanine and tyrosine) and tryptophan were altered in obese Zucker rats, evidenced by lower urinary concentrations of host-microbial co-metabolites including 4-cresyl glucuronide, IS and PAG, and higher fecal 3-HPPA compared to lean animals. 4-Cresol is metabolized from tyrosine *via* 4-hydroxyphenylacetate by the gut microbiota and further metabolized into 4-cresyl glucuronide or 4-cresyl sulfate in the liver (Figure 4). PAG is a conjugation product of glycine and phenylacetate, which is produced by microbiota from phenylalanine, and 3-HPPA is also produced

from phenylalanine[25]. Indoxyl sulfate is produced in the liver from indole which is derived from the microbial metabolism of dietary tryptophan[26]. Our findings indicate reduced microbial activity of producing the precursors of these urinary host-microbial co-metabolites. 4-Cresyl sulfate and phenylacetylglutamine (equivalent to phenylacetylglycine in rodents) have recently been found to be inversely associated with BMI in UK and U.S. cohorts of the INTERMAP (International Study of Macro- and Micronutrients and Blood Pressure) epidemiologic study[27], which are consistent with our findings. A Roux-en-Y gastric bypass surgical model in rats also showed increased urinary concentrations of IS, PAG, 4-cresyl glucuronide and 4-cresyl sulfate after weight loss[28]. Our results indicate that conversion of phenylalanine to 3-HPPA is greater in the obese Zucker rats, whereas in lean animals production of phenylacetate from phenylalanine seems to be enhanced and excreted as PAG in the urine. Higher levels of tyrosine were also taken into the portal vein blood in lean, compared to obese rats. A positive correlation between IS and PAG was observed in both lean and obese rats. In addition, a positive correlation between 4-cresyl glucuronide and PAG or IS was only observed in obese rats, suggesting that in obese animals the metabolism of tyrosine, phenylalanine and tryptophan could be closely inter-linked, whereas in lean rats, 4-cresol production from tyrosine may be dis-proportionally up-regulated in comparison to phenylacetate and indole.

Systemic circulating BCAAs such as valine and isoleucine were higher and fecal BCAAs were lower in the obese compared with lean rats. Elevated plasma BCAAs in obese humans and animals have been reported in previous studies[29]. Wijekoon and colleagues (2004)[29] reported that concentrations of plasma BCAAs were significantly increased in the Zucker obese rat compared to lean rat. Although we did not observe a correlation between BCAAs and fasting insulin levels, a gradual increase in plasma BCAAs of obese and diabetic individuals was reported by Adeva et al (2012)[30] and the study suggested that the metabolism of BCAAs is particularly responsive to the inhibitory insulin action on amino acid release by skeletal muscle and their metabolism is profoundly altered in conditions featuring insulin resistance, insulin deficiency, or both.

Obese Zucker rats showed higher levels of lipids in both portal vein and peripheral blood plasma and higher concentrations of lactate peripheral blood plasma compared with lean rats, which are expected and in agreement with previous studies[6, 31-33]. Glucose is oxidized to pyruvate and the latter is catalyzed by lactate dehydrogenase, yielding lactate and NAD^+ which is further required for the oxidation 3-phosphoglyceraldehyde during production of pyruvate from glucose[34]. In addition, lipid signals ($CH_2C=C$ and CH=CH) in the obese animals were observed to be negatively correlated with D-3-hydroxybutyrate, which is a ketone body produced in the liver mainly from the oxidation of fatty acids and exported to peripheral tissues for use as an energy source[35]. Ketosis occurs as a result of the change in the body's fuel from carbohydrate to fat and has a significant effect on suppressing hunger[36]. The inter-correlation among the metabolites involved in energy metabolism is more prominent in obese animals compared with lean rats, evidenced by strong positive correlation between lipid signals and pyruvate, alanine or lactate. Alanine can be produced from pyruvate *via* alanine transaminase and a positive correlation between alanine and pyruvate was observed in obese rats but not in lean rats. Alanine can also be produced by BCAAs, which may contribute approximately 60% of the nitrogen for alanine synthesis[37]. We observed a negative correlation between alanine and valine and isoleucine, indicating a down-regulated synthesis of alanine from BCAAs in obese rats.

■ CONCLUSIONS

Our study has systematically demonstrated for the first time the metabolic profiles of urine, portal vein and peripheral blood plasma and fecal water of hyperinsulinaemic obese Zucker rats. Metabolic observations of portal vein and peripheral blood plasma profiles in both obese and lean Zucker rats indicated that the absorption of SCFAs, choline and TMA was independent of the Zucker rat phenotypes. However, urinary host-microbial co-metabolites such as PAG, IS and 4-cresyl glucuronide were highly associated with phenotypes, suggesting distinct gut microbial metabolic activities in lean and obese Zucker rats. The study also provided a baseline picture of metabolic profiles of a genetically obese rat model in order to further our understanding of utilizing this animal model for future obesity studies.

■ ASSOCIATED CONTENT

Supporting Information

This section contains the full methods for animal model and sampling, measurement of OGTT, glucose and fasting insulin, fecal water extraction, ^{1}H NMR analysis of biofluids, data pre-processing methods, multivariate data analysis and statistical correlation analysis of biofluids.

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Author Contributions

JVL and FS designed the experiments; FS performed the animal experiment; JP performed metabolic profiling analysis; JP and JVL interpreted the data; JP, FS, HL, JRM, JKN, EH and JVL wrote and edited the manuscript.

Notes

The authors declared no competing financial interest.

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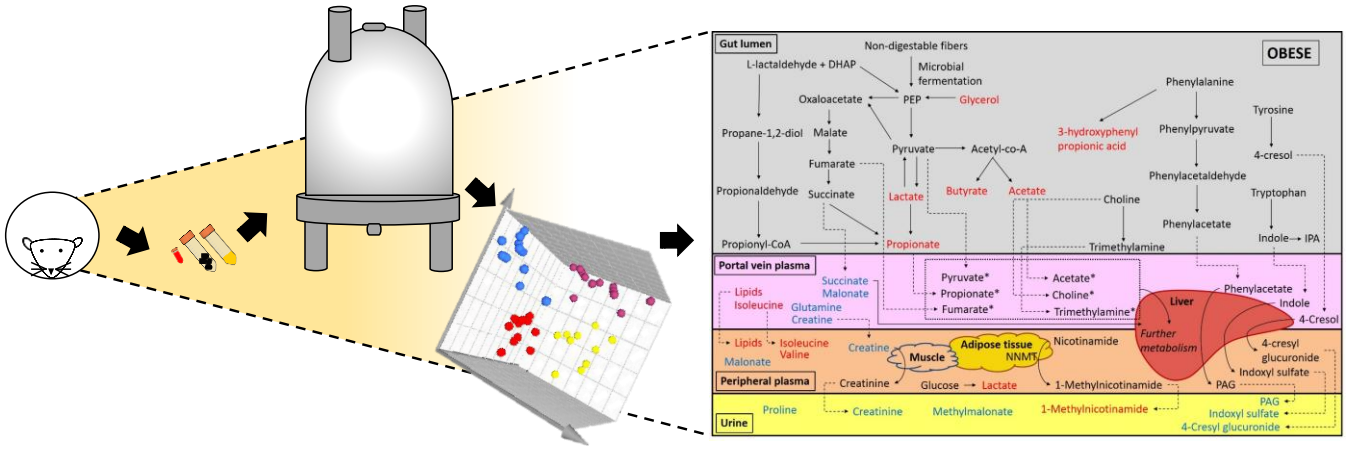
Gut lumen
Non-digestable fibers
OBESE
L-lactaldehyde + DHAP
Microbial fermentation
Phenylalanine
Oxaloacetate
PEP
Glycerol
Tyrosine
Propane-1,2-diol
Malate
3-hydroxyphenyl propionic acid
Phenylpyruvate
Pyruvate
Acetyl-co-A
4-cresol
Fumarate
Phenylacetaldehyde
Propionaldehyde
Succinate
Lactate
Butyrate
Acetate
Choline
Tryptophan
Phenylacetate
Propionyl-CoA
Propionate
Trimethylamine
Indole
IPA
Portal vein plasma
Succinate
Malonate
Pyruvate*
Acetate*
Lipids
Isoleucine
Glutamine
Creatine
Propionate*
Choline*
Phenylacetate
Fumarate*
Trimethylamine*
Liver
Indole
4-Cresol
Lipids
Isoleucine
Valine
Creatine
Adipose tissue
NNMT
Nicotinamide
Further metabolism
4-cresyl glucuronide
Malonate
Muscle
Indoxyl sulfate
Peripheral plasma
Creatinine
Glucose
Lactate
1-Methylnicotinamide
PAG
PAG
Proline
Creatinine
Methylmalonate
1-Methylnicotinamide
Indoxyl sulfate
Urine
4-Cresyl glucuronide