High Cysteine Diet Reduces Insulin Resistance in SHR-CRP Rats

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Summary

Increased plasma total cysteine (tCys) has been associated with obesity and metabolic syndrome in human and some animal studies but the underlying mechanisms remain unclear. In this study, we aimed at evaluating the effects of high cysteine diet administered to SHR-CRP transgenic rats, a model of metabolic syndrome and inflammation. SHR-CRP rats were fed either standard (3.2 g cystine/kg diet) or high cysteine diet (HCD, enriched with additional 4 g L-cysteine/kg diet). After 4 weeks, urine, plasma and tissue samples were collected and parameters of metabolic syndrome, sulfur metabolites and hepatic gene expression were evaluated. Rats on HCD exhibited similar body weights and weights of fat depots, reduced levels of serum insulin, and reduced oxidative stress in the liver. The HCD did not change concentrations of tCys in tissues and body fluids while taurine in tissues and body fluids, and urinary sulfate were significantly increased. In contrast, betaine levels were significantly reduced possibly compensating for taurine elevation. In summary, increased Cys intake did not induce obesity while it ameliorated insulin resistance in the SHR-CRP rats, possibly due to beneficial effects of accumulating taurine.

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Introduction

Cysteine (Cys) is a conditionally essential and potentially obesogenic amino acid which can be obtained from the diet, breakdown of body proteins or synthesized endogenously from methionine (Met) by a sequence of transmethylation and transsulfuration reactions (Olsen

et al. 2018, Brosnan and Brosnan 2006). Cys is the rate-limiting substrate for synthesis of the major intracellular antioxidant glutathione (GSH) and its availability is also essential for synthesis of proteins, coenzyme A, hydrogen sulfide (H_2S) and taurine (Tau). Cys is tightly regulated to maintain its steady-state concentrations below the threshold of cytotoxicity (Stipanuk *et al.* 2006, Stipanuk 2020). Cys is catabolized *via* CDO-dependent pathway or *via* several desulfhydration reactions. The main CDO-dependent catabolic pathway converts Cys to cysteinesulfinate with subsequent channeling into either the decarboxylation route yielding Tau or the transamination pathway that produces inorganic sulfite and sulfate. To a lesser extent Cys is catabolized to H_2S by the cytosolic desulfhydration pathways involving cystathionine beta-synthase, gamma-cystathionase and aspartate aminotransferase with mercaptopyruvate sulfurtransferase. Hydrogen sulfide is subsequently converted by a series of enzymatic reactions located in mitochondria to glutathione persulfide, sulfite, or thiosulfate and sulfate (Fig. 1).
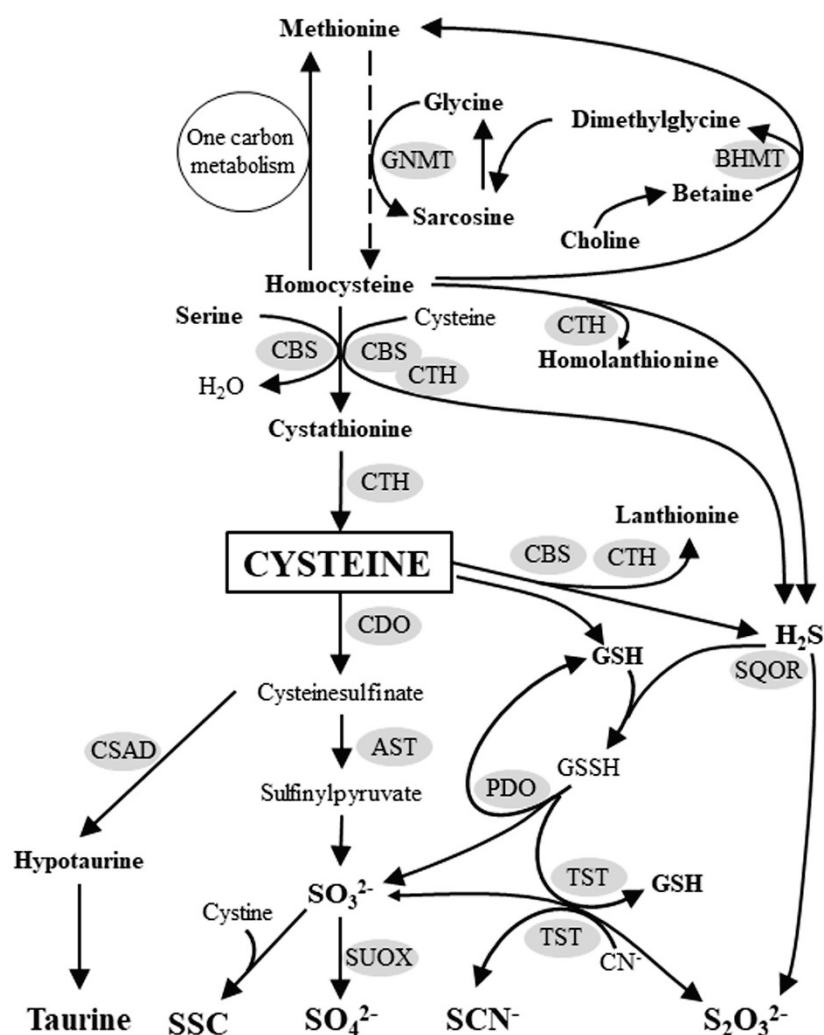

Fig. 1. Cysteine synthesis and catabolism. Cys is produced by transsulfuration pathway from dietary Met and is catabolized either by decarboxylation pathway (end-product taurine), transamination pathway (end-product sulfate) or desulfhydration pathways (product hydrogen sulfide). Hydrogen sulfide is converted by enzymatic reactions located in mitochondria to sulfite, thiosulfate and sulfate. Abbreviations: CBS, cystathionine beta-synthase; CSAD, cysteine sulfinic acid decarboxylase; AST, aspartate aminotransferase; CDO, cysteine dioxygenase; CTH, gamma-cystathionase; GSH, glutathione; GSSH, glutathione persulfide; GNMT, glycine N-methyltransferase; BHMT, betaine:homocysteine methyltransferase; H_2S, hydrogen sulfide; PDO, persulfide dioxygenase; SUOX, sulfite oxidase; SQOR, sulfide:quinonenoxidoreductase; TST, thiosulfate transferase; SO_3^{2-}, sulfite; SO_4^{2-}, sulfate; $S_2O_3^{2-}$, thiosulfate; SCN^-, thiocyanate; SSC, S-sulfocysteine.

A strong direct association between plasma total cysteine (tCys, i.e. the sum of reduced, oxidized and protein bound cysteine) and components of metabolic syndrome such as obesity, BMI, fat mass, insulin resistance, central adiposity and lipid metabolism has been shown in previous studies involving human subjects (Elshorbagy *et al.* 2012c, Elshorbagy *et al.* 2012b, El-Khairy *et al.* 1999, Elshorbagy *et al.* 2008). Moreover, intermediate phenotypes (e.g. activity of the lipogenic enzyme stearoyl-CoA desaturase, fibroblast growth factor 21, IGF-1, serum leptin and triglycerides) are adversely influenced by high-cysteine (HCD) and/or high methionine diet and reversed by sulfur amino acid restriction (Elshorbagy *et al.* 2012d, Olsen *et al.* 2020, Niewiadomski *et al.* 2016, Olsen *et al.* 2018, Elshorbagy *et al.* 2012a). All these findings strongly support the

hypothesis that Cys is causally related to obesity and pathogenesis of metabolic syndrome in humans. In contrast, several studies in rodents indicated beneficial effect of increased Cys intake on metabolic syndrome components. Treatment of Zucker diabetic fatty rats with Cys supplemented diet reduced glycemia, lowered insulin resistance, reduced oxidative stress and decreased levels of inflammatory markers (Jain *et al.* 2009). Similarly, increased Cys intake prevented oxidative stress and development of insulin resistance after feeding rats with high-sucrose diet (Blouet *et al.* 2007). Controversies on the effects of HCD or Cys restricted diet on the components of metabolic syndrome in humans and rodent models raise a question whether the elevated levels of Cys found in obese patients result from excessive dietary intake, increased endogenous Cys production or decreased catabolism. In this study, we hypothesized that high Cys intake would increase adiposity and insulin resistance in a rodent model of metabolic syndrome and inflammation (SHR-CRP strain), and we explored the metabolic and molecular consequences of this dietary intervention.

Methods

Study design

As obesity is associated with chronic inflammation, we studied obesogenic effects of HCD in SHR-CRP strain, a model of metabolic syndrome and inflammation. The experiment was conducted in 3-month-old male SHR-CRP transgenic rats expressing the human CRP gene under control of the apolipoprotein E promoter with the objective of driving expression of the CRP transgene in liver where CRP is normally produced (Pravenec *et al.* 2011). In the current study, we tested the hypothesis that feeding HCD is associated with increased adiposity and insulin resistance as primary outcomes. A total of 16 SHR-CRP rats were divided into two groups and fed with different experimental diets for 4 weeks. The first group was fed standard diet (SD) Altromin 1314 (3.244 g cystine/kg diet, 3.171 g Met/kg diet). The second group received HCD, consisting of Altromin 1314 enriched with 4g L-cysteine/kg diet. The estimated daily intake of Cys, calculated on the expected 28 g daily intake of the diet, was approximately 1.7 and 0.75 mmoles in the HCD and SD fed rats, respectively. The daily Met intake in both diets was 0.6 mmoles. After 4 weeks of dietary treatment, urine was collected, rats were sacrificed by decapitation, and blood and tissues

(liver, kidney, brain, muscle) were collected. Urine was collected only from 12 animals – 6 urine samples from each group. The urine was collected in metabolic cages overnight for 16 h. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic and approved by the Ethics Committee of the Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic (71/2015).

Determination of metabolic syndrome parameters

Basal and insulin-stimulated glucose utilisation in isolated epididymal adipose tissue and muscles. For measurement of insulin-stimulated incorporation of glucose into lipids or glycogen, epididymal adipose tissue or diaphramatic muscle was incubated for 2 h in 95 % O_2 + 5 % CO_2 in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 0.1 µCi/ml of ^{14}C-U glucose, 5 mmol/l of unlabelled glucose and 2.5 mg/ml of bovine serum albumin (Fraction V, Sigma, Czech Republic) with and without 250 µU/ml of insulin. Extraction of lipids or glycogen was followed by a determination of insulin-stimulated incorporation of glucose into lipids or glycogen (Malinska *et al.* 2015).

Tissue triglyceride measurements. For determination of triglycerides in liver, gastrocnemius muscle and heart, tissues were powdered under liquid N_2 and extracted for 16 h in chloroform:methanol, after which 2 % KH_2PO_4 was added and the solution was centrifuged. The organic phase was collected and evaporated under N_2. The resulting pellet was dissolved in isopropyl alcohol, and triglyceride concentrations were determined by an enzymatic assay (Erba-Lachema, Brno, Czech Republic).

Biochemical analyses. Blood glucose levels were measured by the glucose oxidase assay (Erba Lachema, Brno, Czech Republic) using tail vein blood drawn into 5 % trichloroacetic acid and promptly centrifuged. Non-esterified fatty acids levels were determined using an acyl-CoA oxidase-based colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany). Serum triglyceride concentrations were measured by standard enzymatic methods (Erba Lachema, Brno, Czech Republic). Serum insulin and leptin concentrations were determined using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden; MyBioSource, USA).

Parameters of oxidative stress. Oxidative stress was measured according to the activities of antioxidant enzymes, concentrations of reduced and oxidized

glutathione, and levels of lipoperoxidation products thiobarbituric acid-reactive substances (TBARS) (Malinska *et al.* 2015). The activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) were analyzed using Cayman Chemicals assay kits (MI, USA). Catalase (CAT) activity measurement was based on the ability of H_2O_2 and ammonium molybdate to produce a colour complex that was detected spectrophotometrically. Lipoperoxidation products were assessed according to concentrations of TBARS determined by assaying the reaction with thiobarbituric acid. The levels of conjugated dienes were analyzed by extraction from the media into heptane:isopropanol (2:1) and measured spectrophoto-metrically in the heptane layer. Concentrations of reduced (GSH) and oxidised (GSSG) forms of glutathione were determined using a HPLC diagnostic kit with fluorescence detection (ChromSystems, Germany).

Metabolite analyses

Tissue sample preparation: Tissue samples were weighed and homogenized mechanically using Tissue raptor homogenizer (Qiagen) in buffer containing 1.5 % of laurylmaltoside in 100 mM Tris, pH 8.5 while tubes were held in an ice/water slush. For every 10 mg of tissue we used 60 µl of buffer. Tissue homogenates were centrifuged at 4 °C at 15,000× g for 20 min. The supernatant was aliquoted and stored at -80 °C prior to metabolite analyses.

LC-MS/MS methods: Previously published LC-MS/MS method was used for determination of Tau, hypotaurine, choline, betaine, dimethylglycine and S-sulfocysteine (Kozich *et al.* 2019). Commercially available kit was used for LC-MS/MS determination of amino acids Met, lanthionine and sarcosine (EZ:faast, Phenomenex, Torrance, USA). All LC-MS/MS analyses were performed on a system consisting of the Agilent 1290 Infinity LC System (Agilent Technologies, Palo Alto, CA, USA) coupled with an API 4000 triple quadrupole mass spectrometer with an electrospray ion source. Detection of analytes was carried out using positive electrospray ionization technique and selected multiple reaction monitoring.

HPLC and capillary electrophoresis methods: Total aminothiols (Cys, cysteinyl-glycine, homocysteine, glutathione and γ-glutamyl-cysteine) and inorganic sulfur compounds (sulfite, thiosulfate, thiocyanate and hydrogen sulfide) were determined by reversed-phase HPLC with fluorescent detection (Shimadzu LC-20AD system with RF-20AXs fluorescence detector, Shimadzu Corporation,

Kyoto, Japan). Aminothiols were derivatized with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) after the reduction of disulphide bonds with tris(2-carboxyethyl)phosphine (TCEP) as described previously (Krijt *et al.* 2001). Sulfite, thiosulfate and thiocyanate were analyzed after derivatization with monobromobimane as described previously (Kozich *et al.* 2019), bioavailable hydrogen sulfide measurements were carried out as described before in (Ditroi *et al.* 2019). Sulfate was determined by Agilent 7100 capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with a capacitively coupled contactless conductivity detection: 50 µl of plasma sample was mixed with 5 µl of 10 mM potassium chlorate and 145 µl of acetonitrile while for urinary analysis 5 µl of urine was mixed with 50 µl of water, 5 µl of 10 mM potassium chlorate and 140 µl of acetonitrile. The resulting mixtures were centrifuged at 14,500× g for 10 min and supernatant was injected into the unmodified fused-silica capillary (50 µm ID and 375 µm OD; Polymicro Technologies, Phoenix, AZ, USA). Between individual runs the capillary was flushed for 2 min with 1 M NaOH, 1 min with water and 2 min with background electrolyte. Background electrolyte consisted of 1 M formic acid in water. Samples were injected using a pressure of 5 kPa for 5 s. A voltage of -25 kV was applied during the analysis. Conductivity detector provided positive signal for sulfate and chlorate (internal standard). Concentration of sulfate was calculated from the ratio of sulfate and chlorate peak areas. Calibration curves were constructed using standard solutions in water for analysis of urine and plasma spiked with sulfate standard solutions for analysis of plasma.

Assessment of renal handling of metabolites. The fractional excretion (excretional fraction, EF) of metabolites was calculated using the standard formula: $EF_{metabolite}$ [%] = 100 × ($U_{metabolite}$ × $P_{creatinine}$) / ($P_{metabolite}$ × $U_{creatinine}$), where U and P are concentrations of analytes determined in simultaneously obtained urine and plasma samples, respectively. Creatinine in urine and plasma was determined using the automated analyzer Hitachi 902 and the commercially available kit "Creatinine" (Jaffe method, Erba Lachema, Czech Republic) and "Creatinine enzymatic" (Erba Lachema, Czech Republic), respectively.

Gene expression profiling and qPCR studies

RNA isolation: Livers were homogenized in Trizol (ThermoFisher) using TissueLyser LT (Qiagen) at 50 Hz for 5 min. Chloroform was then used for phase separation, and the aqueous phase was mixed 1:1 with

70 % ethanol, applied to RNeasy mini spin columns and purified using RNeasy Plus Mini Kit (Qiagen) according to manufacturer's protocol. RNA was quantified by spectrophotometry.

RNAseq method: We used RNAseq method for determination of gene expression profiles as described (Lunova *et al.* 2021). Library preparation was done with SENSE total RNA AEQ library prep kit for Illumina (Lexogene, PN A01107). Library size distribution was evaluated on the Agilent 2100 Bioanalyzer using the High Sensitivity DNA Kit (Agilent). Libraries were sequenced on the Illumina NextSeq® 500 instrument using 84 bp single-end configuration. Read quality was assessed by FastQC. For subsequent read processing, a bioinformatic pipeline nf-core/rnaseq version 1.4.2, was used. Individual steps included removing sequencing adaptors with Trim Galore, mapping to reference genome Rnor_6.0 (Ensembl annotation version 99) with HISAT2 and quantifying expression on gene level with featureCounts. Per gene mapped counts served as input for differential expression analysis using DESeq2 R Bioconductor package. Prior to the analysis, genes not expressed in at least two samples were discarded. Shrunken log2-fold changes using the adaptive shrinkage estimator were used for differential expression analysis. We supplied experimental model assuming sample treatment as main effect. Genes exhibiting minimal absolute $\log_2$-fold change value of 1 and statistical significance (adjusted p-value<0.05) between compared groups of samples were considered as differentially expressed. Gene set enrichment analysis was done using gene length bias aware algorithm implemented in goseq R Bioconductor package with KEGG pathways and GO terms data.

Quantitative PCR (qPCR). 1 µg total RNA was used to synthesize cDNA using SuperScript IV reverse transcriptase (ThermoFisher) according to manufacturer's protocol. The resulting cDNAs were then used as templates in quantitative real-time PCR (qPCR) reactions. Primers for qPCR reactions (Table S1) were designed using PrimerBLAST (Ye *et al.* 2012) to span at least one exon-exon junction: amplicon size was set to 70-150 bp. Each qPCR reaction contained equivalent of 8 ng of input RNA, 300 nM of each forward and reverse primer and 1× Power-up SYBRGreen master mix (Thermofisher) and was amplified in 7900HT (Applied Biosystems).

For qPCR experiment, cycle threshold (Ct) values of selected genes were normalized relative to the expression of peptidylprolyl isomerase A (*Ppia,* cyclophilin) gene (for liver), which served as the internal control, with results being determined in triplicates. Relative quantification was performed using the ΔΔCt method.

Statistical analyses

The data presented in tables are shown as mean and standard deviation. Outlying values were excluded using Grubbs test. Parametric *t*-test with Benjamini-Hochberg correction for multiple testing was used for comparison of the HCD and SD groups, significance of the false discovery rate (FDR) was set at<0.05. All statistical analyses were performed using Statistica Cz, version 14 (StatSoft, Inc., USA).

Results

Effects of HCD on glucose and lipid metabolism and parameters of oxidative stress and inflammation

Compared to controls on SD the SHR-CRP rats fed a HCD had similar body weight, weight of epididymal and perirenal fat depots, and serum triglyceride levels but exhibited increased serum NEFA concentrations (Table 1). HCD reduced significantly levels of serum insulin. Serum glucose tended to be lower but the difference did not achieve statistical significance. Insulin stimulated adipogenesis in white adipose tissue tended to be increased after HCD but the difference was also not statistically significant. Together these results suggest amelioration of insulin resistance in rats fed HCD. In addition, feeding of HCD reduced oxidative stress as evidenced by significantly lower hepatic concentrations of lipoperoxidation products TBARS and increased activity of GSH peroxidase (Table 2). There were no significant effects of HCD on serum markers of inflammation as MCP-1, IL6, and TNF-α concentrations were similar to controls on SD (data not shown).

Effects of HCD on metabolite profiles

Concentration of Cys in tissues and body fluids

The levels of tCys and related analytes in body fluids and tissue samples from rats on HCD and SD are summarized in Tables 3-4; relative changes in animals fed HCD compared to SD are shown in Table S2. In accordance with previous studies (Stipanuk *et al.* 2009, Stipanuk and Ueki 2011) the HCD did not elicit any significant elevation of tCys in tissues, plasma or urine, demonstrating the tight regulation of Cys homeostasis.

Table 1. Body and organ weights and parameters of glucose and lipid metabolism in SHR-CRP transgenic rats treated with high cysteine diet (HCD) or standard diet (SD).

	SD	HCD
Body weight (g)	392±12	390±18
Weight of epididymal fat (g)	2.55±0.32	2.27±0.82
Weight of perirenal fat (g)	2.09±0.38	2.16±0.41
Liver weight (g)	13.3±0.5	12.7±0.9
Serum glucose (mmol/l)	8.09±0.31	7.67±0.33
Serum insulin (nmol/l)	**0.55±0.18**	**0.31±0.10***
Serum triglycerides (mmol/l)	0.56±0.10	0.56±0.11
Serum NEFA (mmol/l)	**0.50±0.05**	**0.62±0.10***
Serum cholesterol (mmol/l)	1.33±0.14	1.35±0.22
Serum HDL cholesterol (mmol/l)	1.23±0.14	1.29±0.19
Liver triglycerides (µmol/g)	8.85±2.47	8.67±1.31
Muscle gastrocnemius triglycerides (µmol/g)	2.23±0.97	3.75±2.83
Heart triglycerides (µmol/g)	**1.49±0.24**	**0.88±0.14***
Sensitivity of white adipose tissue to insulin action (nmol glucose incorporated/g tissue/2 h)	482±85	782±482

t-test with Benjamini-Hochberg correction; *** bold**, significant difference with FDR<0.05.

Table 2. Parameters of oxidative stress in the liver in SHR-CRP transgenic rats treated with high cysteine diet (HCD) or control diet (SD).

	SD	HCD
SOD (U/mg)	0.13±0.02	0.14±0.03
GSH-Px (µM NADPH/min/mg)	**270±65**	**354±46***
GR (µM NADPH/min/mg)	122±23	133±30
CAT (mM H$_2$O$_2$/min/mg)	1539±355	1704±422
GSH (µmol/mg protein)	56.0±2.2	53.6±2.4
GSSG (µmol/mg protein)	2.16±0.04	1.69±0.21
GSH/GSSG	27.0±5.4	29.9±7.4
Conjugated dienes (nM/mg)	28.4±3.9	24.3±4.1
TBARS (nM/mg)	**1.78±0.48**	**1.17±0.16***

t-test with Benjamini-Hochberg correction; *** bold**, significant difference with FDR<0.05.

Indices of Cys catabolism

Metabolites of the decarboxylation pathway

The end product of the catabolic decarboxylation pathway Tau was significantly increased to ≈1.2-4.5-fold in urine and tissues except the brain and increased to ≈3-fold in the EF of HCD-fed animals. Concentration of the intermediate hypotaurine was also increased to ≈2-fold in urine obtained from rats on HCD.

Metabolites of the cysteine transamination and desulfhydration pathways

Sulfite is produced as an intermediate in the transamination of cysteinesulfinate and the desulfhydration pathways, and its levels in tissues, plasma and urine were not significantly different between rats fed HCD and SD. However, excretion of more stable markers of sulfite accumulation, i.e. S-sulfocysteine, thiosulfate and thiocyanate, was increased to ≈1.3-1.5-fold. Hydrogen sulfide originating from the desulfhydration pathway was elevated to ≈2.3-fold in plasma of HCD fed animals while the byproduct of H$_2$S synthesis – lanthionine – was significantly elevated only in the muscle. Finally, urinary excretion of the final product of sulfite oxidation – sulfate – was significantly increased to ≈1.6-fold by HCD.

Table 3. Concentrations of cysteine and related metabolites in tissues of rats on HCD and SD.

	Concentrations in tissues *(µmol/g protein* (except for ratios))*							
	Liver		**Kidney**		**Brain**		**Muscle**	
	SD	**HCD**	**SD**	**HCD**	**SD**	**HCD**	**SD**	**HCD**
Total cysteine	4.97±2.20	4.58±1.49	23.7±3.4	23.1±1.6	11.8±0.6	10.6±1.4	2.93±2.03	1.82±0.58
Cysteine catabolism − decarboxylation pathway								
Hypotaurine	2.58±1.07	2.65±1.09	2.38±0.55	2.62±0.69	**0.95±0.10***	**1.07±0.10***	4.50±1.16	6.54±2.45
Taurine (Tau)	**20.0±14.1**	**90.4±15.5***	**77.3±5.2**	**95.0±12.0***	**60.5±7.2**	**49.8±8.2***	**358±67**	**536±156***
Cysteine catabolism – transamination pathway								
Sulfite	0.034±0.006	0.036±0.004	0.0018±0.0002	0.0019±0.0002	0.0022±0.0002	0.0022±0.0003	0.013±0.006	0.016±0.006
Cysteine catabolism − desulfhydration and related analytes								
Hydrogen sulfide	0.0068±0.0017	0.0078±0.0017	0.012±0.002	0.014±0.002	NA	NA	NA	NA
Thiosulfate	0.013±0.003	0.012±0.002	0.18±0.03	0.17±0.02	0.016±0.003	0.013±0.003	0.038±0.012	0.038±0.012
Thiocyanate	0.18±0.09	0.15±0.06	1.02±0.20	0.95±0.34	**0.32±0.0**	**0.27±0.04***	0.60±0.14	0.77±0.23
S-sulfocysteine	ND	ND	ND	ND	ND	ND	ND	ND
Lanthionine	ND	ND	0.0073±0.0016	0.0069±0.0012	0.0044±0.0016	0.0048±0.0015	**0.0045±0.0017***	**0.0072±0.0024***
Glutathion metabolism								
Glutathione	40.4±5.5	40.0±2.4	0.12±0.01	0.13±0.01	2.06±0.47	1.76±0.60	16.8±5.9	23.4±9.2
γ-Glutamyl-cysteine	0.055±0.005	0.068±0.017	0.27±0.03	0.29±0.03	0.17±0.01	0.18±0.02	0.0073±0.0016	0.0071±0.0018
Cysteinylglycine	0.36±0.04	0.35±0.02	0.49±0.06	0.51±0.06	0.11±0.02	0.11±0.02	0.21±0.12	0.14±0.04
Methionine cycle and related analytes								
Methionine	0.46±0.07	0.42±0.06	2.67±0.48	2.64±0.45	0.36±0.06	0.34±0.04	0.91±0.24	1.00±0.39
Total homocysteine	0.11±0.01	0.11±0.01	0.28±0.03	0.27±0.01	0.063±0.005	0.053±0.017	0.012±0.004	0.014±0.007
Met/tHcy	4.37±0.62	3.97±0.64	9.58±0.92	9.96±1.75	5.43±0.46	7.11±2.30	68.2±14.7	97.1±70.8
Choline	4.36±0.53	4.72±1.58	24.1±4.5	24.1±3.1	6.52±0.32	6.37±1.10	**0.93±0.13**	**1.84±0.54***
Betaine	**15.6±4.1**	**7.7±1.1***	**18.9±3.7**	**13.6±4.7***	**0.22±0.04**	**0.17±0.05***	4.36±1.34	3.63±1.34
Dimethylglycine	**0.53±0.09**	**0.27±0.05***	**0.072±0.018**	**0.045±0.007***	0.0022±0.0014	0.0012±0.0008	0.14±0.04	0.13±0.05
Sarcosine	0.30±0.08	0.25±0.06	**0.078±0.009**	**0.060±0.009***	0.0068±0.0017	0.0054±0.0012	0.40±0.14	0.40±0.27

t-test with Benjamini-Hochberg correction; *** bold**, significant difference with FDR<0.05.

Table 4. Concentrations of cysteine and related metabolites in body fluids of rats on HCD and SD.

	Plasma		Urinary output		Urine		EF	
	µmol/l		*nmol/h*		*µmol/mmol of creatinine*		*%*	
	SD	**HCD**	**SD**	**HCD**	**SD**	**HCD**	**SD**	**HCD**
Total cysteine	187±12	177±12	44.7±14.7	53.0±10.0	11.1±3.0	11.9±1.9	0.2±0.0	0.2±0.1
Cysteine catabolism − decarboxylation pathway								
Hypotaurine	11.2±2.3	10.5±2.8	**53.7±16.1**	**123±26***	**13.3±2.9**	**27.7±6.0***	**3.2±0.9**	**8.5±3.4***
Taurine (Tau)	262±59	294±33	**1709±501**	**5650±870***	**425±90**	**1265±153***	**4.5±1.2**	**13.2±1.9***
Cysteine catabolism − transamination pathway								
Sulfite	0.022±0.006	0.017±0.003	0.28±0.06	0.27±0.09	0.072±0.023	0.060±0.019	10.0±5.6	10.4±4.6
Sulfate	1068±114	994±86	**11275±2165**	**18813±1138***	**2842±427**	**4231±292***	**7.8±2.3**	**13.6±0.8***
Decarboxylation/Transamination pathways ratio								
Tau/Sulfate	0.25±0.08	0.30±0.06	**0.15±0.03**	**0.30±0.04***	**0.15±0.03**	**0.30±0.04***	NA	NA
Cysteine catabolism − desulfhydration and related analytes								
Hydrogen sulfide	**0.0031±0.0005**	**0.0071±0.0047***	NA	NA	NA	NA	NA	NA
Thiosulfate	1.14±0.30	0.91±0.31	**2.45±0.41**	**3.53±0.44***	**0.62±0.11**	**0.80±0.14***	**1.5±0.5**	**3.3±1.7***
Thiocyanate	**33.6±6.4**	**43.6±8.2***	**25.2±6.8**	**41.3±12.1***	6.65±2.93	9.31±2.94	0.6±0.3	0.7±0.2
S-sulfocysteine	1.80±0.14	1.81±0.76	**2.54±0.56**	**3.83±0.42***	**0.63±0.06**	**0.86±0.12***	**1.0±0.2**	**1.6±0.6***
Lanthionine	0.034±0.005	0.031±0.006	0.068±0.015	0.105±0.046	0.017±0.003	0.024±0.011	1.4±0.4	2.3±1.0
Glutathione metabolism								
Glutathione	**57.7±7.4**	**43.6±13.8***	4.08±0.84	5.13±1.19	1.02±0.12	1.14±0.22	**0.05±0.01**	**0.1±0.1***
γ-Glutamyl-cysteine	5.33±0.53	4.62±0.78	NQ	NQ	NQ	NQ	NA	NA
Cysteinylglycine	1.20±0.15	1.15±0.13	25.6±4.8	30.4±6.7	6.47±1.14	6.81±1.27	15.4±4.2	17.5±2.0
Methionine cycle and related analytes								
Methionine	**23.2±2.8**	**19.0±3.1***	13.1±2.5	14.5±2.7	3.30±0.46	3.22±0.42	0.4±0.1	0.5±0.1
Total homocysteine	**2.71±0.14**	**2.99±0.25***	1.78±0.99	2.21±0.82	0.44±0.21	0.50±0.22	0.5±0.3	0.5±0.3
Met/tHcy	**8.62±1.32**	**6.36±1.14***	8.74±3.44	7.18±2.28	8.74±3.44	7.18±2.28		
Choline	10.1±0.8	11.0±1.1	98.3±33.6	119±34	25.5±10.6	26.6±7.8	7.0±2.8	7.7±3.1
Betaine	**207±16**	**132±16***	**627±156**	**425±51***	**156±22**	**95±10***	2.1±0.3	2.4±0.6
Dimethylglycine	**10.9±0.8**	**6.9±0.5***	257±70	199±21	**63.8±9.6**	**44.9±5.5***	16.2±1.8	18.5±2.0
Sarcosine	**1.73±0.22**	**1.21±0.18***	**29.1±8.9**	**17.8±2.1***	**7.25±1.57**	**4.02±0.58***	11.7±2.1	10.2±1.8

t-test with Benjamini-Hochberg correction; *** bold**, significant difference with FDR<0.05.

Glutathione related metabolites

As Cys is a substrate for GSH synthesis, we evaluated the impact of HCD on the concentrations of GSH and related metabolites. Plasma GSH was decreased to ≈0.8-fold and EF GSH was elevated to ≈2-fold while there were no significant changes in concentrations of intermediates in GSH synthesis (gamma-glutamylcysteine) and degradation product (cysteinylglycine).

Metabolites of the methionine cycle

We observed significantly decreased levels of Met (to ≈0.8-fold) and slightly increased levels of total homocysteine (to ≈1.1-fold) in plasma of HCD rats. The decrease of Met/total homocysteine ratio to ≈0.7-fold may indicate an impaired remethylation of homocysteine to Met. Interestingly, the methyl group donor in one of the two remethylation reactions, betaine, was decreased to ≈0.5-0.8-fold in all analyzed tissues except muscle and in body fluids of rats on HCD. In addition, the betaine demethylation metabolites – dimethylglycine and sarcosine – were also decreased to ≈0.5-0.8-fold in liver, kidney, plasma and urine of HCD fed rats. On the other hand, the precursor of betaine synthesis – choline – was

significantly elevated (to ≈2-fold) only in muscles of rats on HCD.

Effects of HCD on hepatic gene expression profiles

Using RNAseq we observed significantly deregulated expression levels of 22 transcripts (Table S3). Data were subsequently evaluated by gene enrichment analysis using KEGG pathways map. SHR-CRP rats fed a HCD had significantly increased hepatic expression of circadian rhythms pathway genes *Per1*, *Per3* and *Nr1d1* and reduced expression of *Cxcl10* and *Rnf125* genes in the RIG-I-like receptor signaling pathway suggesting reduced inflammation. In addition, the RNAseq analysis revealed suggestive deregulated expression of *Csad* (cysteinesulfinic acid decarboxylase), *Slc6a6* (solute carrier family 6 member 6, also known as taurine transporter) and *Mthfr* (methylenetetrahydrofolate reductase) genes. However, qPCR testing of selected genes involved in sulfur metabolism confirmed only significantly downregulated expression of the *Slc6a6* gene after HCD while deregulation of the *Csad* and *Mthfr* genes did not reach statistical significance (Fig. 2).
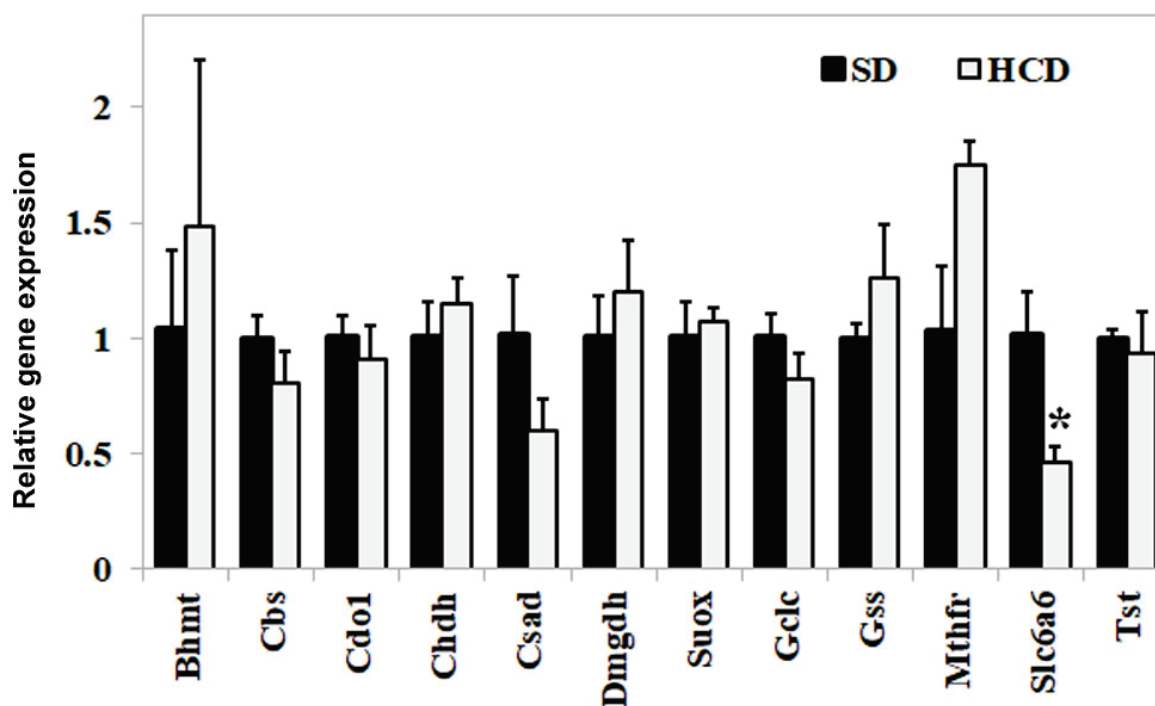

Fig. 2. Hepatic expression of selected genes in sulfur metabolic pathways. The open bars indicate relative expression of genes analyzed by qPCR in animals on HCD. Expression of genes on SD was set to 1.0, Differences between animals on HCD and SD were evaluated by *t*-test with Benjamini-Hochberg correction, * indicates significant difference; Abbreviations: *Bhmt*, betaine:homocysteine methyltransferase; *Cbs*, cystathionine beta-synthase; *Cdo1*, cysteine dioxygenase; *Chdh*, choline dehydrogenase; *Csad*, cysteine sulfinic acid decarboxylase; *Dmgdh*, dimethylglycine dehydrogenase; *Suox*, sulfite oxidase; *Gclc*, glutamate-cysteine ligase, catalytic subunit; *Gss*, glutathione synthase; *Mthfr*, methylenetetrahydrofolate reductase; *Slc6a6*, solute carrier family 6 member 6; *Tst*, thiosulfate transferase.

Discussion

Previous studies consistently showed that elevated plasma tCys is associated with several components of metabolic syndrome in humans (Elshorbagy *et al.* 2012c, Elshorbagy *et al.* 2012b, El-Khairy *et al.* 1999, Elshorbagy *et al.* 2008). Moreover, reduced sulfur amino acid intake had beneficial effect on circulating markers of metabolic syndrome and gene expression in adipose tissues (Olsen *et al.* 2020). In the present study we used an established rodent model of metabolic syndrome and inflammation, the SHR-CRP rats, to explore the hypothesis that increased Cys intake induces obesity and increases insulin resistance. In contrary, our study showed that feeding the SHR-CRP animals a diet enriched with Cys did not increase obesity while it ameliorated insulin resistance and decreased oxidative stress. Although some previous studies reported impairment of metabolic syndrome on HCD (Elshorbagy *et al.* 2012a), results of our study are similar to previous reports of beneficial effect of Cys supplementation on markers of oxidative stress and components of metabolic syndrome (Jain *et al.* 2009, Blouet *et al.* 2007). However, since we did not measure food intake it may be possible that the lack of effect of the HCD on body weight/fat mass could be due to unrecognized effects of the diet on caloric intake. Higher Cys intake did not change concentrations of tCys in tissues and body fluids, and resulted in elevation and decrease of Tau and betaine, respectively, and changes in expression of circadian rhythms and inflammation related genes.

We estimated that the daily intake of sulfur amino acids in the HCD and SD fed rats was ≈2.3 mmoles and ≈1.3 mmoles, respectively. Despite ~70 % higher sulfur amino acid load in HCD fed rats we did not observe any increase of tCys in tissue, plasma or urine. Previous studies of rodents on HCD also demonstrated such tight regulation of Cys homeostasis, which is likely mediated by abundance and/or activity of CDO, CSAD and glutamate-cysteine ligase (GCL) (Stipanuk 2020). In our study, RNAseq and qPCR analyses did not reveal any significant changes in *Cdo1* gene expression in liver from HCD-fed rats despite clearly increased urinary losses of the end products of the CDO-mediated catabolic pathways. This agrees with previous studies showing that changes in gene expression probably play a minor role in regulating CDO activity in Cys overload. CDO is activated mainly post-transcriptionally *via* formation of a cysteinyl-tyrosine

crosslink in the active site of CDO enzyme and by inhibited degradation *via* the ubiquitin-proteasome system (Dominy *et al.* 2006, Stipanuk *et al.* 2009). In contrast to previous studies in rats on HCD (Vidal *et al.* 2014) we did not observe any significant changes in GSH and GSH-related metabolites or differences in hepatic expression of *Gclc.*

Cys is catabolized *via* two CDO-dependent pathways (decarboxylation and transamination) and several reactions in the desulfhydration pathway. Data from our study allow us to roughly estimate the diversion of Cys among these routes. Although we were unable to determine the unstable intermediates cysteinesulfinate and sulfinylpyruvate in the decarboxylation and transamination pathways, these CDO-dependent pathways appear to be the major routes for removing excess Cys. The daily urinary output of Tau increased by ≈0.1 mmoles/day while sulfate excretion rose by ≈0.2 mmoles/day, suggesting that partitioning of cysteinesulfinate between the decarboxylation and transamination pathways favors transamination with a caveat that an unknown proportion of sulfate may originate from oxidation of sulfite that is generated *via* desulfhydration of Cys. Partitioning of cysteinesulfinate between decarboxylation and transamination is modulated by CSAD. Indeed, we observed a decreased expression (albeit not significant with the p=0.096) of *Csad* in the livers of HCD rats which is similar to previous reports (Bella *et al.* 1999, Jurkowska *et al.* 2016). The desulfhydration reactions producing H_2S appear to play a lesser role in maintaining Cys homeostasis and become more important only in CDO deficiency or Cys starvation when CDO levels are very low (Stipanuk and Ueki 2011, Stipanuk 2020). In our study, H_2S concentration was not increased in tissues but it was doubled in plasma of HCD-fed rats; however, the latter data should be interpreted with caution due to possible preanalytical intricacies of collecting blood after decapitation. Lanthionine, a marker of Cys desulfhydration, was not significantly elevated in body fluids and tissues except muscle, further supporting a lower flux *via* the desulfhydration pathway. However, higher flux of Cys *via* both the transamination and desulfhydration pathways that generate sulfite is also indirectly indicated by an increased urinary excretion of S-sulfocysteine, thiosulfate and thiocyanate.

Extensive metabolite profiling revealed also unexpected changes in the remethylation pathway of homocysteine. We observed decreased concentration of

the methyl group donor betaine and its demethylated derivatives – dimethylglycine and sarcosine – in tissues and body fluids. The origin of these changes remains obscure as the hepatic expression of genes involved in betaine synthesis and metabolism – choline dehydrogenase, betaine aldehyde dehydrogenase, dimethylglycine dehydrogenase, sarcosine dehydrogenase – did not differ between HCD and SD rats except for increased expression of the *Bhmt* gene in HCD, however, the difference was not statistically significant. Lower concentration of betaine may in turn decrease the supply of methyl groups for the BHMT-dependent conversion of homocysteine to Met as indicated by the decreased Met/total homocysteine ratio in liver.

The beneficial effects of HCD on metabolic syndrome components may be in part mediated by increased concentrations of Tau (Imae *et al.* 2014). Previous reports showed that Tau supplementation decreased weight gain and adiposity in mice (Tsuboyama-Kasaoka *et al.* 2006), reduced insulin resistance and blood glucose levels, and decreased serum levels of triglycerides and leptin in a model of type 2 diabetes rats (Kim *et al.* 2012) and inhibited insulin stimulated H_2O_2 release and activation of lipolysis in adipocytes (Pina-Zentella *et al.* 2012). In addition, elevated Tau concentrations were previously shown to downregulate expression the *Slc6a6* gene that encodes the taurine transporter (TauT) with subsequently decreased differentiation of adipose-derived stem cells to adipocytes *via* a beta-catenin related mechanism (Hou *et al.* 2019). The important role of TauT in adipogenesis was shown in the *Slc6a6* knockout mice that exhibited lower body weight and abdominal fat mass (Ito *et al.* 2015). In our study, decreased hepatic expression of *Slc6a6* gene in SHR-CRP animals on HCD was not accompanied by decreased adiposity or liver triglyceride concentration, however, triglycerides content was significantly reduced in the myocardium.

Other mechanisms may be mediated by deregulated expression of genes in the circadian rhythms and RIG-I-like receptor signalling pathways, respectively. *Per1* (period circadian regulator 1), *Per3* (period circadian regulator 3) and *Nr1d1* (nuclear receptor subfamily 1, group D, member 1) genes play important role in regulating circadian rhythms and metabolism. For instance, knock-out of *Nr1d1* gene in mice was associated with hepatic steatosis (Na *et al.* 2016). In addition, *Nr1d1* ablation in mice suppressed glycolysis and fatty acid oxidation in white adipose tissue (WAT), whereas partial

Nr1d1 loss in heterozygotes, curiously stimulated these processes. Thus, *Nr1d1* dose-dependently regulates glucose metabolism and fatty acid oxidation in WAT and muscle (Welch *et al.* 2020). Both *Cxcl10* (C-X-C motif chemokine ligand 10) and *Rnf125* (ring finger protein 125) genes play important role in innate immunity and are downregulated in rats fed HCD which suggests reduced inflammation.

Conclusions

In conclusion, this study showed various effects of HCD on the metabolic and molecular outcomes in SHR-CRP rats. Some of these effects (e.g. the lack of obesogenic effects and amelioration of insulin resistance in rats fed HCD) seem to contradict the hypothesis that Cys promotes obesity and metabolic syndrome in humans. It is possible that these positive effects of HCD on selected metabolic syndrome parameters result from elevated Tau levels, which may protect against obesity and insulin resistance. These results in a rodent model, however, are not necessarily in conflict with the established association of tCys with components of metabolic syndrome in humans. It is possible that increased tCys levels detected in obese individuals may result not only from an increased Cys intake but also from impaired Cys catabolism. Defect in CDO activity, which may lead to higher Cys levels and reduced levels of the metabolic syndrome ameliorating compound Tau may be an example of another mechanism that increases tCys in humans with metabolic syndrome. Further studies are needed to evaluate these hypotheses.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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Supplementary Table 1. Primers for qPCR reactions.

Bhmt_e7F	GACATGCACACCAAACCCTG
Bhmt_e8R	AAGGATTGTACGGTCTGCCC
Cbs_e13F	CCTCCGGGAGAAGGGTTTTG
Cbs_e14R	CATGTTCCCGAGAGTCACCA
Cdo1_e4F	GCCTTTGACCAACGAACAGG
Cdo1_e5R	CTTTGAAAGGCTTGGCAGGTC
Chdh_e4F	CTGCTCATGCTCTCTGGTGT
Chdh_e5R	GTCCTGCAGGTTCTGACCAA
Csad_e5F	TGGGGATGGGGTCTTCTGT
Csad_e7R	CTTGGTGATGGAGTAGTGGCA
Dmgdh_e9F	GGAACAGCCACACTGGTTCTA
Dmgdh_e10R	ACTTGCCAAACGGAGACAGG
Suox_e1Fb	CAATGCTGCCTCGGCTATACA
Suox_e2Rb	CTCAAGGGGGTTGACTTGGC
Gclc_e15F	GTGGACACCCGATGCAGTAT
Gclc_e16R	TCATCCACCTGGCAACAGTC
Gss_e8F	AGACCAAAACCGAAGGCTGT
Gss_e9R	GGCATGTAGCCATCTCGGAA
Mthfr_e10F	GAGAAACCGTGGAGGCTCTT
Mthfr_e11R	CAGCTCAGGGGCATTAGTGAT
Slc6a6_e11F	AGGAAGGGTTATCGTCGGGA
Slc6a6_e12R	GCGACCCACAAAAGGCATAC
Tst_e1F	CTCAAGACCTATGAGCAGGTCC
Tst_e2R	ACGTTGACAGAGCCACGGAT
Ppia_c59F	TGTTCTTCGACATCACGGCT
Ppia_c178R	ATCCTTTCTCCCCAGTGCTC

Supplementary Table 2. Fold changes in metabolite concentrations in animals on HCD versus SD.

	LIVER	KIDNEY	BRAIN	MUSCLE	PLASMA	Urinary output	URINE	EF
Analyte fold difference – HCD versus SD								
Total cysteine	0.92	0.97	0.90	0.62	0.95	1.19	1.07	1.00
Methionine cycle related analytes								
Methionine	0.91	0.99	0.94	1.10	**0.82***	1.11	0.98	1.25
Total homocysteine	1.00	0.96	0.84	1.17	**1.10***	1.24	1.14	1.00
Met/tHcy	0.91	1.04	1.31	1.42	**0.74***	0.82	0.82	-
Choline	1.08	1.00	0.98	**1.98***	1.09	1.21	1.04	1.10
Betaine	**0.49***	**0.72***	**0.77***	0.83	**0.64***	**0.68***	**0.61***	1.14
Dimethylglycine	**0.51***	**0.63***	0.55	0.93	**0.63***	0.77	**0.70***	1.14
Sarcosine	0.83	**0.77***	0.79	1.00	**0.70***	**0.61***	**0.55***	0.87
Cysteine catabolism – decarboxylation pathway analytes								
Hypotaurine	1.03	1.10	**1.13***	1.45	0.94	**2.29***	**2.08***	**2.66***
Taurine	**4.52***	**1.23***	**0.82***	**1.50***	1.12	**3.31***	**2.98***	**2.93***
Cysteine catabolism – transamination pathway analytes								
Sulfite	1.06	1.06	1.00	1.23	0.77	0.96	0.83	1.04
Sulfate	NA	NA	NA	NA	0.93	**1.67***	**1.49***	**1.74***
Decarboxylation/Transamination pathways ratio								
Tau/sulfate					1.20	**2.00***	**2.00***	
Cysteine catabolism – desulfhydration and related analytes								
Hydrogen sulfide	1.15	1.17	NA	NA	**2.29***	NA	NA	NA
Thiosulfate	0.92	0.94	0.81	1.00	0.80	**1.44***	**1.29***	**2.20***
Thiocyanate	0.83	0.93	**0.84***	1.28	**1.30***	**1.64***	1.40	1.17
S-sulfocysteine	ND	ND	ND	ND	1.01	**1.51***	**1.37***	**1.60***
Lanthionine	ND	0.95	1.09	**1.60***	0.91	1.54	1.41	1.64
Glutathion metabolism analytes								
Glutathione	0.99	1.08	0.85	1.39	**0.76***	1.26	1.12	**2.00***
γ-glutamylcysteine	1.24	1.07	1.06	0.97	0.87	ND	ND	NA
Cysteinylglycine	0.97	1.04	1.00	0.67	0.96	1.19	1.05	1.14

NA, not analysed, ND, not determined, below the LLQ, The differences were calculated from the corresponding means in the rat group on HCD and SD; ***** **bold**, significant difference.

Supplementary Table 3. Significantly deregulated genes in Liver from rats on HCD or SD.

Gene	Name	Fold change	P value	Corrected P value
Dbp	D-box binding PAR bZIP transcription factor	2.89	1.47e-39	1.53e-35
Mfsd2a	major facilitator superfamily domain containing 2A	1.63	3.51e-34	1.82e-30
Il6r	interleukin 6 receptor	1.09	4.15e-22	1.44e-18
Por	cytochrome p450 oxidoreductase	1.02	1.79e-18	4.32e-15
Usp2	ubiquitin specific peptidase 2	1.55	2.47e-18	4.32e-15
Per1	period circadian regulator 1	1.50	2.49e-18	4.32e-15
Nr1d1	nuclear receptor subfamily 1, group D, member 1	1.01	1.46e-17	2.17e-14
Herpud1	homocysteine inducible ER protein with ubiquitin like domain 1	1.11	3.08e-16	3.56e-13
Rnf125	ring finger protein 125	-1.18	6.00e-14	6.23e-11
Per3	period circadian regulator 3	2.20	2.34e-12	2.03e-09
Osgin1	oxidative stress induced growth inhibitor 1	-1.18	3.34e-12	2.67e-09
Creld2	cysteine-rich with EGF-like domains 2	1.05	5.15e-12	3.82e-09
RGD1562310	RIKEN cDNA 2410131K14 gene	1.10	3.38e-11	2.19e-08
Angptl4	angiopoietin-like 4	-1.00	1.67e-10	8.91e-08
Cxcl10	C-X-C motif chemokine ligand 10	-1.12	8.41e-10	4.16e-07
Ciart	circadian associated repressor of transcription	2.46	1.01e-09	4.75e-07
Srms	src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites	-1.25	9.98e-09	3.58e-06
Cib2	calcium and integrin binding family member 2	-1.17	1.27e-08	3.96e-06
Zfp354a	zinc finger protein 354A	1.64	1.29e-08	3.96e-06
Nim1k	NIM1 serine/threonine protein kinase	-1.30	1.01e-07	2.47e-05
Mybl1	MYB proto-oncogene like 1	-1.01	1.02e-07	2.47e-05
Gtse1	G-2 and S-phase expressed 1	1.16	1.52e-07	3.50e-05