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Multifactorial modulation of susceptibility to L-lysine in an animal model of glutaric aciduria type I 2

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ABSTRACT

Glutaric aciduria type I is an inherited defect in L-lysine, L-hydroxylysine and L-tryptophan degradation caused by deficiency of glutaryl-CoA dehydrogenase (GCDH). The majority of untreated patients presents with accumulation of neurotoxic metabolites - glutaric acid (GA) and 3-hydroxyglutaric acid (3-OHGA) - and striatal injury. Gcdh^{-/-} mice display elevated levels of GA and 3-OH-GA but do not spontaneously develop striatal 23 lesions. L-lysine-enriched diets (appr. 235 mg/d) were suggested to induce a neurological phenotype similar to 24 affected patients. In our hands 93% of mice stressed according to the published protocol remained asymptomatic. 25 To understand the underlying mechanism, we modified their genetic background (F1 C57BL6/Jx129/SvCrl) and 26 increased the daily oral L-lysine supply (235-433 mg). We identified three modulating factors, (1) gender, (2) ge-27 netic background, and (3) amount of L-lysine. Male mice displayed higher vulnerability and inbreeding for more 28 than two generations as well as elevating L-lysine supply increased the diet-induced mortality rate (up to 89%). 29 Onset of first symptoms leads to strongly reduced intake of food and, thus, L-lysine suggesting a threshold for 30 toxic metabolite production to induce neurological disease. GA and 3-OH-GA tissue concentrations did not cor- 31 relate with dietary L-lysine supply but differed between symptomatic and asymptomatic mice. Cerebral activities 32 of glyceraldehyde 3-phosphate dehydrogenase, 2-oxoglutarate dehydrogenase complex, and aconitase were 33 decreased. Symptomatic mice did not develop striatal lesions or intracerebral hemorrhages. We found severe 34 spongiosis in the hippocampus of Gcdh^{-/-} mice which was independent of dietary L-lysine supply. In conclusion, 35 the L-lysine-induced pathology in $Gcdh^{-/-}$ mice depends on genetic and dietary parameters. 36

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1. Introduction 42

The mitochondrial homotetrameric flavoprotein glutaryl-CoA dehy-43drogenase (GCDH, EC 1.3.99.7) is required for oxidative decarboxylation 44 of glutaryl-CoA to crotonyl-CoA in the final degradative pathways of the 4546amino acids L-lysine, L-hydroxylysine, and L-tryptophan [1-3]. Quantitatively, L-lysine is the major precursor of glutaryl-CoA. Autosomal reces-47 sive GCDH deficiency due to two disease-causing mutations in the GCDH 48 49 gene (gene map locus: 19p13.2) causes glutaric aciduria type I (GA-I), a cerebral organic aciduria biochemically characterized by accumulation 50of the dicarboxylic metabolites glutaryl-CoA, glutaric acid (GA), 3-5152hydroxyglutaric acid (3-OH-GA), and glutarylcarnitine (C5DC) [4,5]. 53Two biochemical groups - so-called high and low excreters - have

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been arbitrarily delineated based on highly variable GA excretion in 54 patients with or without residual enzyme activity [6,4,7]. The overall 55 estimated incidence of GA-I is about 1 in 100.000 newborns [8], but 56 might be as high as 1 in 200 newborns in high-risk populations [9–13]. 57 Patients are usually asymptomatic at birth or show minor symptoms 58

such as axial muscular hypotonia or asymmetric posturing but if un- 59 treated are at high risk to develop irreversible striatal injury between 60 age 3 to 36 months [14,15] and, subsequently, a complex movement 61 disorder with predominant secondary dystonia superimposing on 62 axial hypotonia [16–18,12]. The onset of striatal injury often manifests 63 acutely during an acute encephalopathic crises which is precipitated 64 by catabolism due to infectious diseases, however, may also occur insid- 65 iously without such crises [19,20]. There is no known genotype-pheno- 66 type correlation [21]. The natural history may be highly variable, even in 67 siblings and in patients with the same GCDH gene mutations [8] sug- 68 gesting an impact of modifier genes and environmental factors on the 69 clinical phenotype. The only known correlation is between the genotype 70 and the biochemical phenotype [7]. Patients with a low excreting phe-71 notype due to high residual GCDH activity have the same risk for 72

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developing striatal injury as high excreting patients with complete loss 73 74 of GCDH activity [17]. This is puzzling since at high concentrations glutaryl-CoA, GA, and 3-OH-GA become neurotoxic via inhibition of 2-7576 oxoglutarate dehydrogenase complex [22] and the dicarboxylic acid shuttle [23,24] as well as stimulation of N-methyl-D-aspartate receptors 77 [25], and increased production of reactive oxygen species [26,27]. The 78 79striking similarity in the disease course of high and low excreters was 80 suggested to be due to similarly high cerebral concentrations of GA 81 and 3-OH-GA concentrations in both patient groups [28–32]. Cerebral 82 entrapment of these dicarboxylic acid in the brain compartment occurs 83 due to a lacking high capacity transport system for dicarboxylic acids in the blood-brain barrier (BBB) [21,33,34]. 84

Striatal injury can be prevented in the majority of patients by 85 86 early identification by newborn screening and immediate start of a combined metabolic treatment with low L-lysine diet, carnitine sup-87 plementation and intermittent glucose infusion or carbohydrate-88 rich, L-lysine-free diet during episodes that are likely to induce catab-89 90 olism [35-43]. Low L-lysine diet and glucose infusion are thought to be most effective in lowering cerebral concentrations of GA and 3-91 OH-GA [44]. This effect was also demonstrated in $Gcdh^{-/-}$ mice 92[45,46], a transgenic animal model for GA-I with complete loss of Gcdh 93 94 activity biochemically resembling patients with a high excreting pheno-95type [47,33]. Supplementation with L-arginine (or homoarginine) which competes with L-lysine for transport across biological membranes such 96 as the BBB (CAT1 transporter) and the inner mitochondrial membrane 97(ORNT1 transporter) further reduces the cerebral L-lysine influx and 98 thus decreases the cerebral concentrations of GA and 3-OH-GA [45,46]. 99 100 In analogy, complementary dietary treatment using L-lysine-free, arginine-fortified amino acid mixtures has been associated with a 101 favorable outcome in prospectively followed newborn screening cohorts 102 103 [38,48].

 $Gcdh^{-/-}$ mice on a C57BL/6Crl \times 129/SvCrl background do not spon-104105taneously develop a neurological phenotype resembling GA-I, nor can it be precipitated by various means of inducing catabolism [47]. Exposure 106 of $Gcdh^{-/-}$ mice on the same genetic background to high protein or 107 high L-lysine diet (L-lysine content in both diets, 4.7%), however, was 108 shown to induce seizures, paralysis, subarachnoidal hemorrhages, and 109110 death within 3-6 days in the majority of 4-week-old mice, but not in 8-week-old mice suggesting age-dependent susceptibility similar to 111 the window of vulnerability for striatal injury in GA-I patients [49,46]. 112 Interestingly, the source of L-lysine, protein bound or free amino acids 113 in chow, did not affect the induced phenotype in these studies. 114

A subgroup of 4-week-old $Gcdh^{-/-}$ mice with less strongly elevated 115 GA concentrations, however, remained asymptomatic for unknown rea-116 sons. This is similar to the known variable clinical phenotype in siblings 117 and patients with the same GCDH gene mutation. Naturally occurring 118119 susceptibility to L-lysine or protein (mice) or catabolism (human) suggests an impact of modifier genes and/or environmental factors on the 120clinical phenotype. The major aim of our study was to validate and to 121optimize the oral L-lysine loading model for Gcdh^{-/-} mice elucidating 122factors that modify the susceptibility of $Gcdh^{-/-}$ mice to the L-lysine-123124induced phenotype. These factors must be controlled to establish a 125reliable animal model, but may also help to unravel new therapeutic strategies for this disease. 126

127 2. Materials and methods

128 2.1. Animals

Mice used in this study had a C57BL/6Crl \times 129/SvCrl background. Inbreeding of these mice from F1–F8 was used to alter the variability of the genetic background along the generations. Animal breeding and experiments were approved by the governmental review board (Regierungspräsidium Karlsruhe, Germany; No. 35-9185.81/G-72/10).

2.2. Treatment

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The high L-lysine diet contained 4.7% (w/w) of L-lysine (Harland 135 Teklad, Indianapolis, IN), the standard diet contained 1.7% (w/w) of L-136 lysine (Rod18, Las Vendi). To identify age-dependent changes in the susceptibility to high L-lysine diet, $Gcdh^{-/-}$ mice received high L-138 lysine or standard diet for a maximum of two weeks starting at ages of 3, 4, 5, 6, or 8 weeks. Concentration-dependent effects of L-lysine exposure were studied by varying the L-lysine concentrations of the drinking water (from 0 to 6.6% [w/v]) in addition to fixed L-lysine contents (1.7 versus 4.7% [w/w]) in chow. Healthy $Gcdh^{-/-}$ and $Gcdh^{+/-}$ mice showed an average daily intake of 4 ml drinking water and 3.5 g chow the per mouse. Table 1 illustrates the daily L-lysine supply per mouse and day in our experiments.

To test the impact of genetic background on the susceptibility to L-lysine, $Gcdh^{-/-}$ mice (C57BL/6 Crl × 129/SvCrl) inbred for up to 8 generations were exposed to increasing amounts of this amino acid in chow 149 and drinking water. As an additional control, we used C57BL/6J mice exposed to the same experimental conditions. $Gcdh^{-/-}$ mice have been 151 generated by injecting ES cells derived from 129X1/SvJ mice into 152 blastocytes from C57BL/6J mice and chimeric male animals were 153 crossed to C57Bl/6J females [47]. Thereafter, these mice were crossed 154 to 129X1/SvJ generating the reported (C57BL/6 Crl × 129/SvCrl) back-55 ground [22,46,47,49].

2.3. Preparation of subcellular fractions and homogenates

For biochemical analyses, symptomatic mice were sacrificed shortly 158 before death and asymptomatic mice after two weeks of treatment. All 159 mice were decapitated and perfused with a solution of phosphate buffremoved and chilled on ice in a buffer (0.1 ml per 1 mg of tissue) containing 250 mmol/l sucrose, 50 mmol/l KCl, 5 mmol/l MgCl₂, and 20 mmol/l Tris–HCl (adjusted to pH 7.4). Homogenates and mitochondrial and cytosolic subcellular fractions of tissues were prepared as described before [45]. Protein concentrations were determined according to Lowry [50] with modifications [51] using bovine serum albumin as a standard.

2.4. Quantitative analysis of GA and 3-OH-GA

The tissue-specific concentrations of GA and 3-OH-GA were deter- 170 mined in tissue homogenates using quantitative gas chromatography/ 171 mass spectrometry with stable-isotope dilution assay as previously 172 described [33]. 173

2.5. Quantitative analysis of C5DC 174

C5DC concentrations were determined in tissue homogenates 175 (600 \times g supernatant) and serum by electrospray ionization tandem 176 mass spectrometry according to a previously described method [33]. 177

2.6. Amino acid analysis

Amino acid content of brain homogenates was analyzed by highperformance liquid chromatography as previously described [45]. 180

Table 1 Amount of L-lysine supplied by chow ($\%$ w/w) and drinking water in ($\%$ w/v) and in mg per mouse and day based on average intake of 3 ml drinking water and 3.5 g chow.									
L-lysine (chow %)	1.7	4.7	4.7	4.7	4.7	4.7	t1.4		
L-lysine (water %)	0	0	1.5	3	4.7	6.5	t1.5		
L-lysine [mg/day and mouse]	85	235	280	325	376	433	t1.6		

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181 2.7. Enzyme analysis

Steady-state activity of enzymes involved in respiratory chain,
 glycolysis, and tricarboxylic acid cycle (TCA) was determined using a
 computer-tuneable spectrophotometer (SpectramaxPlus Microplate
 Reader, Molecular Devices; Sunny Vale, CA) operating in the dual wave length mode; samples were analyzed in temperature-controlled 96 well plates in a final volume of 300 µl.

188 2.8. Spectrophotometric analysis of glycolytic enzymes

Enzyme activities in cytosolic fractions were analyzed with and without addition of substrate to subtract unspecific background. Glycolytic enzyme activities were determined as NADP or NAD reduction, or NADH oxidation at $\lambda = 340-400$ nm as previously described [52].

193 2.9. Electron transport chain (ETC) and electron flux

Respiratory chain complexes I–IV and ATP synthetase in mitochondria fractions were investigated as previously described [53,44,52]. The addition of standard respiratory chain inhibitors was used to ascertain the specificity of the enzymatic assays. To study electron flow from complex I or II to complex III mitochondrial fractions were incubated with NADH or succinate and, subsequently, the reduction of cytochrome c was analyzed in the presence of NaCN.

201 2.10. Spectrophotometric analysis of TCA enzymes

202 *Aconitase* activity was determined according to [22], with modifica-203 tions. Aconitase (0.70 mg) was assayed in a buffer containing 0.7 U IDH, 204 36 mmol/Tris–HCl, 0.07 mmol/l citric acid, 0.18 mmol/l NADP, 1.3 205 mmol/l manganese sulfate, 0.8 µmol/l ferrous ammonium sulfate, and 206 0.08 mmol/l L-cysteine which was adjusted to pH 7.4 (25 C). Aconitase 207 activity was determined as NADP reduction at $\lambda = 340-400$ nm.

208 2-Oxoglutarate dehydrogenase complex (OGDHc) activity was mea-209 sured according to [22], with modifications. OGDHc (650 mU/ml) was 210 assayed in a buffer containing 35 mmol/l potassium phosphate, 5 211 mmol/l MgCl₂, 0.5 mmol/l EDTA, 0.5 mmol/l NAD, 0.2 mmol/l thiamine 212 pyrophosphate, 0.04 mmol/l CoA-SH, and 2 mmol/l 2-oxoglutarate 213 which was adjusted to pH 7.4 (30 °C). OGDHc activity was determined 214 as NAD reduction at $\lambda = 340-400$ nm.

215 *Citrate synthase* activity was measure as described previously [22].

216 2.11. Histology

For histological studies, mice were sacrificed at identical time points 217218as for biochemical analyses, and brains were perfused with 4% paraformaldehyde and post-fixed for 24 h in this solution and embedded in 219paraffin. Paraffin blocks were cut in slices of 3-4 µm thickness using a 220microtome (Leica Microsystems, Nussloch GmbH, Nussloch, Germany) 221and placed onto SuperFrost slides (Thermo Scientific, Dreieich, 222223 Germany). Sections were stained with hematoxylin & eosin (HE), 224Klüver–Barrera (KB), and Periodic acid–Schiff (PAS) according to rou-225tine protocols for histologic evaluation. A spongiosis score was applied taking into account the degree of tissue alteration (0 = absent; 1 =226mild; 2 =moderate; 3 =severe). 227

228 2.12. Statistical analysis

229Data are expressed as mean \pm SD unless otherwise stated. Experi-230ments were performed at least in triplicates. Tissue concentrations of231metabolites and enzyme activities were normalized to the protein con-232tent and analyzed by Student's *t*-test or ANOVA. To evaluate the effect of233increased L-lysine exposure on survival, binary logistic regression ana-234lyzed was applied. These statistical analyses were performed using235SPSS for Windows 16.0 Software. Histopathological changes were

analyzed by the non-parametric Wilcoxon test using JMP 8.0.1 software 236 (SAS, Cary, NC, USA). p < 0.05 was considered significant. 237

3.1. Diet-induced mortality is modulated by age, genetic background, and 239 the initial oral L-lysine dose 240

It has been described previously that 75% of weanling (4-week-old) 241 $Gcdh^{-/-}$ mice on a C57BL/6J × 129/SvEv background (first generation) 242 which received a high L-lysine diet (chow with 4.7% L-lysine [w/w], L-ly- 243 sine-free drinking water) died within 3-6 days after the start of this 244 treatment, whereas all adult (8-week-old) mice survived [46]. First, 245 we performed experiments using the original conditions to investigate 246 whether L-lysine-induced mortality was similar in our hands. We con- 247 firmed that L-lysine exposure of 4-week-old $Gcdh^{-/-}$ mice induced a 248 clinical phenotype rapidly progressing to death, but only in a small sub- 249 group (7%). First symptoms were observed as early as 24 h after the 250 start of treatment. The clinical presentation of symptomatic mice 251 started with reduced spontaneous activity and hypothermia and 252 progressed from reduction of food intake, to weight loss (Fig. 1), sei- 253 zures and, subsequently, death. All 8-week-old mice survived and all 254 $Gcdh^{+/-}$ mice (at any age) which were used as controls survived. Com- 255 pared to the original publication [46], the rate of mortality was much 256 lower in our hands (7% versus 75%). 257

We observed that $Gcdh^{-/-}$ mice changed their eating, but not their 258 drinking behavior during high L-lysine exposure. Therefore, we decided 259 to apply additional L-lysine via drinking water. Calculating the actual 260 daily 1-lysine intake based on the amount of chow eaten per mouse, 261 we found that $Gcdh^{-/-}$ mice received relevant amounts of L-lysine 262 only during the first 24 h of treatment [lysine intake in % of symptomatic 263 but living $Gcdh^{-/-}$ mice on the same diet (Median, Min, Max) 24 h: 264 28.2, 0, 51.7; 48 h: 0, 0, 32.9; 96 h: 0, 0, 0]. Thereafter, chow and, 265 hence, L-lysine intake was minimal due to onset of first symptoms indi- 266 cating that the initial L-lysine excess rather than chronic intake was 267 harmful to $Gcdh^{-/-}$ mice. Unlike $Gcdh^{-/-}$ mice on a hybrid background 268 (i.e., C57BL/6J \times 129/SvEv), all 4-week-old *Gcdh*^{-/-} mice on a C57BL/6J 269 inbred background remained asymptomatic and survived the 14-day L- 270 lysine exposure. This suggests that the genetic background modulates 271 the L-lysine-induced mortality. In contrast to $Gcdh^{-/-}$ mice, all control 272 mice ($Gcdh^{+/-}$ mice) survived, irrespective of their genetic background, 273 the amount of daily L-lysine supply and age at start of treatment. In 274 summary, we showed that L-lysine-induced mortality was specifically 275 induced in $Gcdh^{-/-}$ mice, but depended on age and the genetic back- 276 ground. Furthermore, we elucidated that oral lysine loading via chow 277 alone was not reliable, since L-lysine-treated mice changed their eating 278 behavior during exposure.

To better understand the factors modulating L-lysine-induced mor- 280 tality and to optimize the L-lysine exposure model we (1) modified 281



Fig. 1. Body weight under high L-lysine diet. (A) Symptomatic $Gcdh^{-/-}$ mice receiving 285 mg L-lysine per day continuously lost weight, whereas the body weight of control mice remained unchanged (n = 9 mice per group).

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the L-lysine content in drinking water (0-6.5% [w/v]; total L-lysine sup-282 283 ply, 235-433 mg/d, (2) extended the age spectrum from three to eight weeks, and (3) investigated susceptibilities in female and male mice. 284 285Using binary logistic regression analyses we identified three main effects modulating L-lysine-induced mortality in $Gcdh^{-/-}$ mice, i.e. 286(1) gender, (2) the genetic background, and (3) the amount of daily L-287lysine supply (Nagelkerke's $r^2 = 0.624$). Of note, we found no interac-288tion between these factors. Male mice showed a higher vulnerability 289 290 to a high L-lysine exposure than female mice (Fig. 2A; Wald = 8.6, p <0.005). Inbreeding $Gcdh^{-/-}$ mice for at least three generations in-291 creased their vulnerability to a high lysine diet (Fig. 2B; Wald = 28.9, 292p < 0.001) and abolished their lack of susceptibility to a diet containing 293235 mg/d L-lysine. Escalating the daily L-lysine supply up to 433 mg/d 294also increased mortality in treated mice (Fig. 2C; Wald = 8.7, p < 2950.05). As described before [49], 8-week-old mice survived a diet con-296 taining 235 mg/d L-lysine. However, mortality could be induced in 297 these mice by increasing dietary L-lysine content to 325 mg/d (data 298 299not shown). For mice younger than 8 weeks we found no effect of age on the L-lysine-induced phenotype. 300



Fig. 2. Stacked column chart of contingency tables for binary logistic regression analysis. (A) Male mice showed a higher vulnerability to a high L-lysine diet than female mice. (B) Inbreeding *Gcdh^{-/-}* mice for at least 3 generations resulted in an increased vulnerability to L-lysine enriched diet. (C) Mortality rate of *Gcdh^{-/-}* mice was also elevated by increasing the daily L-lysine supply up to 433 mg/d.

In summary, we showed that L-lysine induced mortality is also de- 301 pendent on the genetic background of $Gcdh^{-/-}$ mice. The variation 302 caused by the genetic background, however, can be overcome by in- 303 creasing the amount of daily L-lysine supply of $Gcdh^{-/-}$ mice. Moreover, 304 the gender also modulates the induced phenotype. 305

3.2. Correlation between dicarboxylic compounds and the clinical 306 presentation 307

Since L-lysine is thought to be the major amino acid precursor for 308 toxic dicarboxylic metabolites in GA-I and $Ccdh^{-/-}$ mice, we hypothe-309 sized that the dose-dependent increase in mortality after high L-lysine 310 exposure was associated with a dose-dependent increase in cerebral 311 and hepatic concentrations of toxic GA and 3-OH-GA. In fact, cerebral 312 and hepatic concentrations of both GA and 3-OH-GA increased during 313 L-lysine exposure above that of mice receiving the standard diet 314 (i.e., 85 mg/d). However, cerebral and hepatic concentrations of 315 GA reached a plateau between 235 and 376 mg/d L-lysine and 316 those of 3-OH-GA between 325 and 376 mg/d L-lysine (Fig. 3A,B). 317 Similarly, L-lysine concentrations of brain and liver tissue also 318 reached a plateau at a daily L-lysine supply of 325 mg/d (L-lysine 319 concentration (nmol/mg): 85 mg/d L-lysine supply, brain 11 \pm 8, liver 320 15 \pm 9; 325 mg/d L-lysine supply, brain 25 \pm 4, liver 54 \pm 16).

Next, a statistical comparison of all tested control mice (i.e. Gcdh 322 mice receiving standard diet) with all tested asymptomatic and symp- 323 tomatic $Gcdh^{-/-}$ mice (i.e. $Gcdh^{-/-}$ mice receiving a high lysine diet 324 showing no symptoms and symptoms respectively) was performed. 325 As expected asymptomatic and symptomatic $Gcdh^{-/-}$ mice had higher 326 cerebral and hepatic GA concentrations than control mice. More impor- 327 tantly, symptomatic $Gcdh^{-/-}$ mice could be distinguished from asymp- 328 tomatic mice by a more pronounced increase in GA concentrations in 329 brain and liver (Fig. 3C). For 3-OH-GA, cerebral but not hepatic concen- 330 trations differed from asymptomatic mice, whereas asymptomatic mice 331 displayed the same concentrations in both organs as controls (Fig. 3D). 332 These results support the neurotoxicological hypothesis of GA-I and 333 confirm previous results [25,48,49,46]. To gain a reliable L-lysine-induc- 334 ible clinical phenotype, we performed all following experiments in 4-335 week-old Gcdh^{-/-} mice on a C57BL/6 Crl \times 129/SvCrl inbred back- 336 ground using chow (4.7%, w/w) and drinking water (4.7%, w/v; total 337 L-lysine supply, 376 mg/d) enriched with L-lysine unless otherwise $_{338}$ stated. 339

3.3. Ammonia detoxification remains unaffected during L-lysine exposure 340

Glutaryl-CoA inhibits the TCA cycle [22], whereas GA [24] and – to a 341 lesser extent - 3-OH-GA [23] both inhibit the dicarboxylic acid shuttle 342 between astrocytes and neurons which relies on highly active pyruvate 343 carboxylase in astrocytes and effective sodium-dependent dicarboxylic 344 acid carriers 1 and 2. As a consequence, the flux and availability of TCA 345 cycle intermediates is thought to be impaired in GA-I [33,48]. One im- 346 portant TCA cycle intermediate is 2-oxoglutarate. This metabolite is a 347 substrate of many enzymes such as the 2-oxoglutarate dehydrogenase 348 complex in the TCA cycle. In addition, it provides the carbon backbone 349 for the synthesis of L-glutamine by L-glutamate dehydrogenase and L- 350 glutamine synthetase and thus for the fixation of ammonia. 2- 351 Oxoglutarate is also required as a substrate for the formation of 352 saccharopine within the so-called saccharopine pathway of L-lysine ox- 353 idation [45]. The formation of saccharopine is the first irreversible step 354 of L-lysine oxidation via this pathway. Since it was suggested that L- 355 lysine exposure might limit the availability of 2-oxoglutarate in 356 $Gcdh^{-/-}$ mice [46] and that 3-OH-GA and – less pronounced – GA 357 might increase the concentration of ammonia in 3D-organotypic rat 358 brain cell cultures [54], we wondered whether L-lysine exposure 359 would affect ammonia detoxification and the formation of L-glutamine 360 and urea in $Gcdh^{-/-}$ mice. Hyperammonemic crises are known to 36107

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Fig. 3. Changes in metabolic parameters following high L-lysine exposure. GA levels (A) were elevated in $Gcdh^{-/-}$ mice receiving 235 mg L-lysine per day. Increasing the dietary L-lysine supply above 235 mg/d did not further increase the GA concentrations. The same pattern was found for 3-OH-GA (B) except that 325 mg L-lysine per day enhanced formation of this metabolite (ANOVA with contrasts, *p \leq 0.05). Next, we compared the cerebral and hepatic concentrations of GA (C) and 3-OH-GA (D) in symptomatic [-/- Lys (symptomatic); n = 14] and asymptomatic [-/- Lys (asymptomatic); n = 18] $Gcdh^{-/-}$ mice receiving high L-lysine diet as well as in $Gcdh^{-/-}$ mice on a standard diet [-/- standard diet; n = 9]. GA and 3-OH-GA and 3-OH-GA than asymptomatic mice displayed higher cerebral and hepatic levels of GA and 3-OH-GA than asymptomatic mice (ANOVA, *p \leq 0.05).

induce severe cerebral injury in intoxication type metabolic disorders
 such as urea cycle disorders, but have never been reported in patients
 with GA-I.

365Serum and brain ammonia concentrations did not differ in $Gcdh^{-/-}$ 366 and $Gcdh^{+/-}$ mice after L-lysine exposure (mean \pm SD: serum 105 \pm 38 μ mol/l [*Gcdh*^{-/-}] vs. 105 ± 28 μ mol/l [*Gcdh*^{+/-}]; brain 115 ± 15 μ mol/l 367 $[Gcdh^{-/-}]$ vs. 147 \pm 18 µmol/l $[Gcdh^{+/-}]$), whereas serum urea concen-368 trations were higher in $Gcdh^{-/-}$ (183 \pm 89 mg/dl; p = 0.036) than in 369 $Gcdh^{+/-}$ mice (33 \pm 8 mg/dl). In analogy, urea concentrations in 370brain and liver of $Gcdh^{-/-}$ mice were also elevated (Fig. 4A). Uremia 371due to renal failure was excluded by analysis of serum creatinine and 372cystatin C which both remained in the normal range (creatinine, 373 $Gcdh^{+/-}$ 0.5 \pm 0.4 mg/dl, $Gcdh^{-/-}$ 0.9+/-0.5 mg/dl; cystatin C, 374 $Gcdh^{+/-}$ 0.3 \pm 0.5 mg/l, $Gcdh^{-/-}$ 0.1 +/- 0.1 mg/l). In contrast to 375urea, L-glutamate which is used for transient fixation of ammonia 376 by L-glutamine synthetase in liver and brain remained unchanged 377 in both tissues (Fig. 4B). Similarly, tissue-specific L-glutamine con-378 centrations also remained unchanged following L-lysine exposure 379 380 (Fig. 4C). These results show that L-lysine exposure induces enhanced but effective detoxification of ammonia via the urea cycle, but do not 381support the notion of hyperammonemia playing an important role in 382 the neuropathogenesis of GA-I. The most likely cause of increased 383 ammonia is induction of catabolism following L-lysine exposure. 384

385 3.4. Changes in energy metabolism: focus on glycolysis and the TCA cycle

Previous studies have highlighted that impairment of brain energy metabolism and formation of reactive oxygen species can be induced by accumulating toxic dicarboxylic metabolites and thus is thought to play a key role in the pathogenesis of GA-I [55,48,27]. So far, OGDHc and the dicarboxylate shuttle have been identified as major targets 390 [23,22,24]. We therefore wondered whether L-lysine exposure resulted 391 in changes of the major routes of energy metabolism. 392

L-lysine exposure induced changes in the activity of some glycolysis 393 and TCA cycle enzyme. Specifically, the activities of GAPDH and - less 394 pronounced - of PFK2, PGM2, PK LA, and LDH were specifically de- 395 creased in the brain but not in the liver of $Gcdh^{-/-}$ mice compared to 396 control mice, whereas hexokinase activity was slightly increased in 397 the liver (Table 2A). In the TCA cycle, activities for aconitase (liver and 398 brain) and OGDHc (liver) were lower in $Gcdh^{-/-}$ mice compared to 399 $Gcdh^{+/-}$ mice (Table 2B). In contrast, citrate synthase activity did not 400 differ between $Gcdh^{-/-}$ and $Gcdh^{+/-}$ mice (Table 2B) suggesting that 401 the decreased energy supply via glycolysis and TCA cycle is not 402 counterbalanced by increased mitochondrial proliferation. The activities 403 of respiratory chain enzyme complexes I-IV and ATP synthase remained 404 unchanged (Table 2C). In comparison to a previous study focusing on di- 405 rect metabolite-enzyme interactions [22], these findings do not reflect 406 toxic metabolite-induced enzyme inhibition, but adaptive changes fol- 407 lowing L-lysine exposure. 408

3.5. Hippocampal pathology is most prominent but is not influenced by 409 L-lysine exposure 410

The main neuropathological finding in symptomatic GA-I patients is 411 striatal necrosis which may manifest acutely during encephalopathic 412 crisis or insidiously in infancy [29,20]. In addition, extrastriatal changes 413 have been found both in symptomatic and asymptomatic patients. 414 This includes putatively reversible temporal hypoplasia and dilated 415 Sylvian fissures, T₂ hyperintensity of white matter changes reflecting 416 spongiform myelinopathy as well as T₂ hyperintensities in dentate 417

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Fig. 4. Effect of L-lysine exposure on urea, glutamate, and glutamine. (A) $Gcdh^{-/-}$ mice (-/- Lys, n = 8) showed an increase in urea concentrations of brain and liver compared to controls (+/- Lys, n = 10) after treatment with high L-lysine diet (Student's *t*-test, *p \leq 0.005). Glutamine (B) and glutamate (C) levels were similar in both groups.

418 nuclei, substantia nigra, and the pontine medial lemniscus [56,14,57, 58]. In Gcdh^{-/-} mice receiving high protein or high L-lysine diet, the 419 striatal pathology was thought to be similar to GA-I patients [49,46]. 420 In these studies, however, striatal cell loss was only significantly in-421 creased in asymptomatic 8-week-old animals after a high L-lysine diet 422 423 for 6 weeks but not in symptomatic 4-week-old animals after 3 days of L-lysine exposure. Both treatment groups, however, showed pro-424 nounced cortical cell loss and, additionally, subarachnoidal, subdural 425426 and intraventricular hemorrhages as well as BBB breakdown. Subarachnoidal and intraventricular hemorrhages have not yet been reported in 427GA-I patients, whereas subdural hygromas resembling non-accidental 428head trauma are sometimes found, usually as an incidental finding in 429 430MRI studies [14.15].

In our hands, symptomatic $Gcdh^{-/-}$ mice receiving optimized high L-lysine diet as described in detail above did not produce a significant striatal pathology (Fig. 5). Furthermore, we did not find evidence for BBB breakdown or intracranial hemorrhages (not shown). In contrast to GA-I patients, the major pathology in $Gcdh^{-/-}$ mice was identified in the hippocampus and – less pronounced – in the cortex showing 436 massive focal spongiosis and focal neuronal damage within these 437 areas. Neuronal damage consisted among other features of neuronal 438 pyknosis, nuclear chromatin condensation, and dark neurons indicating 439 very early (i.e. 1–48 h) signs of neuronal damage. These changes, how- 440 ever, appeared in both $Gcdh^{-/-}$ mice on a standard and on a high L- 441 lysine diet.

Statistical analysis confirmed that hippocampal spongiosis was in- 443 creased in $Gcdh^{-/-}$ mice compared to $Gcdh^{+/-}$ mice, but that it did 444 not depend on the amount of L-lysine in the diet (Fig. 5). In analogy to 445 previous studies, we also found an increased rate of spongiosis in the 446 cortex (Fig. 5). No striking pathology was observed in the white matter. 447

4. Discussion

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The major aim of our study was to validate and to optimize the previously published L-lysine-induced mouse model for GA-I [46] and to identify modifiers that influence the induced clinical phenotype. High L-lysine exposure in weanling (4-week-old) but not in adult (8-weekold) $Gcdh^{-/-}$ mice induced an acute clinical phenotype, but only in small group of animals. Initial symptoms are reduced spontaneous activity and hypothermia that progress over weight loss and seizures to death within 24–96 h. Despite these similarities to previous findings [46], we observed significant obstacles in the reliability and reproducibility of this diet-induced model and raised questions on whether the induced neuropathology is similar to that of GA-I patients. In the following, we will discuss our major findings that support the need of a critical revision of this model.

4.1. Genetic background

In our study the mortality rate of $Gcdh^{-/-}$ mice on a high L-lysine 463 diet was strongly dependent on the genetic background. Mice of the 464 F1 generation (C57Bl6/Crlx129/SvCrl; as used in the study of [49,46] 465 as well as mice of F2 generation on a 4.7% (i.e., 235 mg/d) L-lysine diet 466 had 93% survival rate. In contrast, inbreeding of $Gcdh^{-/-}$ mice strongly 467 increased their susceptibility to L-lysine exposure and improved the re- 468 liability of the L-lysine-induced phenoytpe. $Gcdh^{-/-}$ mice of F6–8 gen- 469 erations showed neurological symptoms and a high mortality (~71%) 470 receiving 235 mg L-lysine/d. These findings suggest that susceptibility 471 to high L-lysine diet is based on modifier genes of mice on a mixed 472 C57Bl6/Crl \times 129/SvCrl background whose phenotypic impact changes 473 during inbreeding. Whether increased susceptibility to L-lysine expo- 474 sure from the F1 to the F8 generation reflects an increasing impact of 475 genes which amplify L-lysine-induced toxicity or the decreasing impact 476 of neuroprotective factors remain to be elucidated. Nevertheless, it is 477 well known that inbreeding generally decreases the fitness of mice 478 and their susceptibility to different stress conditions (the so-called in- 479 breeding depression). 480

Moreover, we demonstrate that male mice have a higher risk to de- 481 velop neurological symptoms than female mice on a high L-lysine diet. 482 This is similar to a variety of murine models for neurological disorders 483 such as stroke [59]. 484

4.2. L-lysine exposure

Susceptibility to a high L-lysine diet (235 mg/d) in F1 and F2 gener- 486 ations could be increased by escalating the daily L-lysine supply (280– 487 433 mg/d). All *Gcdh^{-/-}* mice of F6–8 generation receiving at least 488 376 mg L-lysine per day developed neurological symptoms and died 489 within 96 h, whereas 22% of F1–2 generation mice survived on this 490 diet. Since L-lysine exposure induced a decreased food intake, we hy- 491 pothesize that the peak L-lysine intake during the first 24 h of the start 492 of L-lysine exposure is most important for inducing a clinical phenotype 493

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t2.1 Table 2

t2.2 Tissue-specific effects of L-lysine exposure on glycolysis, TCA cycle, and respiratory chain.

+ Q1 Activities of enzymes of energy metabolism in control and $Gcdh^{-/-}$ mice receiving a modified high L-lysine diet (n = 10 per group).

13ble 2.4 Gynelpsin Table 2.4 Gynelpsin Gale 1 4.9 ± 0.8 7.4 ± 0.9 14 Gale 1 4.9 ± 0.8 7.4 ± 0.9 137 Prophofunctokinase Gale 1 4.9 ± 0.8 7.4 ± 0.9 138 Prophofunctokinase Gale 1 3.8 ± 1.9 0.00 139 Tinosphoaphate komerase Gale 1 8.92 ± 227.6 7.06 ± 10.5. 1314 Tinosphoaphate komerase Gale 1 8.92 ± 227.6 7.06 ± 10.5. 1314 Gale 1 8.92 ± 227.6 7.06 ± 10.5. 7.01 ± 10.0.7 1314 Gale 1 8.92 ± 227.6 7.06 ± 10.5. 7.01 ± 10.0.7 1314 Gale 1 2.94 ± 15.3 3.82 ± 16.4 3.82 ± 16.4 1314 Prophofunctokingenase Gale 1 2.94 ± 15.3 3.83 ± 16.1 1314 Prophofunctokingenase Gale 1 2.94 ± 15.3 3.83 ± 16.1 1314 Prophofunctokingenase Gale 1 2.94 ± 15.3 3.81 ± 16.1 1314 Prophofunctokingenase Gale 1 2.94 ± 15.3 <th>t2.4</th> <th></th> <th></th> <th>Brain [mU/mg protein]</th> <th>Liver [mU/mg protein]</th>	t2.4			Brain [mU/mg protein]	Liver [mU/mg protein]
noIterakinaseCach ^ ~ ~4.9 ± 1.26.3 ± 1.1100pValue (Arest)0.8810.0007.4 ± 0.09101pValue (Arest)0.8810.00030.9 ± 1.0101pValue (Arest)0.0120.0120.012101pValue (Arest)0.0120.0120.012101pValue (Arest)0.0127.0 ± 1.0101pValue (Arest)0.0127.0 ± 1.0101pValue (Arest)0.0127.0 ± 1.0101pValue (Arest)0.0127.0 ± 1.0101pValue (Arest)0.0120.005101pValue (Arest)0.0120.005101pValue (Arest)0.0120.005101pValue (Arest)0.0050.701101pValue (Arest)0.0050.701101pValue (Arest)0.0050.701101pValue (Arest)0.0050.701101pValue (Arest)0.0050.701101pValue (Arest)0.0050.701101pValue (Arest)0.0050.701101pValue (Arest)0.0050.701101pValue (Arest)0.0040.007102pValue (Arest)0.0040.007103http://miniformcdn ^{1-/-} 1.012104pValue (Arest)0.0180.012104pValue (Arest)0.0180.012103http://miniformcdn ^{1-/-} 1.012104pValue (Arest)0.018 <td>t2.5</td> <td>Table 2A. Glycolysis</td> <td></td> <td></td> <td></td>	t2.5	Table 2A. Glycolysis			
$2-7$ -8 Allow (rivers) 0.88 $7.4 + 0.9$ 200 Prosphofuctokinase $Calh$ ^{-/-} 2.5 ± 1.3 4.10 ± 8.4 210 $Calh$ ^{-/-} 2.5 ± 1.3 4.10 ± 8.4 110 $Calh$ ^{-/-} 8.22 ± 227.6 770.6 ± 126.5 111 $Cach$ ^{-/-} 8.22 ± 227.6 770.6 ± 126.5 111 $Cach$ ^{-/-} 8.22 ± 227.6 770.6 ± 126.5 111 $Cach$ ^{-/-} 8.24 ± 15.3 8.22 ± 22.6 110 $Cach$ ^{-/-} 8.24 ± 15.3 8.22 ± 2.6 $Cach$ ^{-/-} 140.4 ± 7.22 40.5 ± 3.5 8.22 ± 2.6 $Cach$ ^{-/-} 140.4 ± 7.22 40.5 ± 3.5 8.22 ± 2.6 $Cach$ ^{-/-} 140.4 ± 7.22 40.5 ± 3.5 8.22 ± 2.6 $Cach$ ^{-/-} 140.4 ± 7.22 40.5 ± 3.5 7.5 ± 3.5 1100 $Cach$ ^{-/-} 130.4 ± 7.22 40.5 ± 3.5 7.5 ± 3.5 1100 $Cach$ ^{-/-} 27.3 ± 1.52 56.12 ± 113.2 56.12 ± 113.2 1100 $Cach$ ^{-/-} 27.3 ± 1.52 56.12 ± 113.2 56.12 ± 113.2	t2.6	Hexokinase	Gcdh ^{+/-}	4.9 ± 1.2	6.3 ± 1.1
$ \begin{array}{c c c c c } & \begin{tabular}{c c c c c } & \begin{tabular}{c c c c c } & \begin{tabular}{c c c c c c c } & \begin{tabular}{c c c c c c c c } & \begin{tabular}{c c c c c c c c c c c c c c c c c c c $	t2.7		Gcdh ^{-/-}	4.9 ± 0.8	7.4 ± 0.9
120 Prosphorhurcakinace Coh ⁺ 2.5 ± 1.3 4.30 ± 8.4 1210 Gold*** 1.8 ± 0.0 20.1 ± 1.0 20.00 1211 Priorephosphate isomerac Gold*** 0.032 0.00 1213 Priorephosphate isomerac Gold*** 0.032 1.00 1.00 1214 Priorephosphate isomerac Gold*** 0.034 1.01 1.00 1213 Corealdelyid*3-phosphate delydrogenase Gold*** 1.04 ± 3.72 40.5 ± 4.3 2.01 2.42 ± 1.33 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 2.01 ± 3.03 </td <td>t2.8</td> <td></td> <td>p-Value (<i>t</i>-test)</td> <td>0.881</td> <td>0.000</td>	t2.8		p-Value (<i>t</i> -test)	0.881	0.000
12.10 Cdh ⁻⁺ 13.± 0.9 330.± 12.0 12.11 Picklin (feast) 0.032 2227.6 770.5 ± 12.5 × 12.11 Cdh ⁺⁻ 802.2 ± 227.6 × 770.5 ± 12.5 × 12.11 Cdh ⁺⁻ 802.2 ± 227.6 × 770.5 ± 12.5 × 12.11 Cdh ⁺⁻ 20.49 ± 15.3 × 81.2 ± 2.6 × 12.11 Cdh ⁺⁻ 20.49 ± 15.3 × 81.2 ± 2.6 × 12.12 Picklin (feast) 0.001 0.0880 12.13 Picklin (feast) 0.001 0.0880 12.13 Picklin (feast) 0.001 0.0880 12.14 Picklin (feast) 0.001 0.015 12.14 Picklin (feast) 0.004 0.005 12.14 Picklin (feast) 0.004 0.004 12.14 Picklin (feast) 0.004 0.004 12.14 Picklin (feast) 0.004 0.057 12.14 <	t2.9	Phosphofructokinase	Gcdh ^{+/-}	2.5 ± 1.3	43.0 ± 8.4
11.1 p Value (i.eks) 0.032 0.739 1213 Tosephosphate isomerase 6ch ¹⁺ 832, 1:84.5 77.14 ± 100.7 1213 Gach 833, 1:84.5 73.14 ± 100.7 1213 Gach 833, 1:84.5 73.14 ± 100.7 1214 Gach 146.4 ± 72.2 40.5 ± 4.3 1214 Polaue (ices) 0001 00880 1214 Biosphoghyterate mutase 6ch ⁺ 27.2 ± 3.5.3 207.5 ± 3.0.3 1214 Biosphoghyterate mutase 6ch ⁺ 27.5 ± 3.6.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3 207.5 ± 3.0.3	t2.10		Gcdh ^{-/-}	1.8 ± 0.9	39.0 ± 12.0
11 Coh ^{m-1} 892.2 ± 27.6 710.6 ± 126.5 121 Gohdmann Status 710.6 ± 126.5 121 Gohdmann Status 710.6 ± 126.5 121 Gohdmann Status 110.7 121 Gohdmann Status 103.4 121 Gohdmann Status 114.4 121 Polatic (Lexit) 0001 00580 121 Polatic (Lexit) 0001 00580 121 Polatic (Lexit) 0005 0701 122 Enolate Gohdmann Status Status 122 Enolate Gohdmann Status 703.3 ± 101.3 Status 122 Function form Gtatus 703.3 ± 101.3 Status Status 123 Function form Gtatus 703.3 ± 101.3 Status Status 124 Function form Gtatus Formal Status Status Status 124 Function form Gtatus Formal Status Status Status 124 Formal Gtatus <t< td=""><td>t2.11</td><td></td><td>p-Value (<i>t</i>-test)</td><td>0.032</td><td>0.1790</td></t<>	t2.11		p-Value (<i>t</i> -test)	0.032	0.1790
12.13 $Gah^{}$ 84.3 ± 1.9.5 75.1 ± 100.7 12.14 Gyeenale(hyde 3-phosphate dehydrogenase $Gah^{}$ 10.4 ± 1.3.2 0.5.4 12.14 Gyeenale(hyde 3-phosphate dehydrogenase $Gah^{}$ 10.4 ± 1.3.2 0.5.4 12.15 Phosphoglycente mutase $Gah^{}$ 242.2 ± 3.3.3 21.1 ± 4.0.3 12.16 Phosphoglycente mutase $Gah^{}$ 242.2 ± 3.3.3 21.1 ± 4.0.3 12.21 Phosphoglycente mutase $Gah^{}$ 33.1 ± 1.5.2 56.3 ± 12.3.3 12.22 Dolose $Gah^{}$ 33.1 ± 1.5.2 56.3 ± 12.3.3 12.23 Pyrnvate kinase $Gah^{}$ 33.1 ± 1.5.2 56.3 ± 12.3.3 12.24 Mith filmit form $Gah^{}$ 37.3 ± 1.0.3 84.4 ± 186.1 12.24 Pyrnvate kinase Gah^{+} 37.3 ± 1.0.3 84.4 ± 186.1 12.24 Walke (text) 0.344 64.64 0.327 86.1 12.24 Value (text) 0.344 64.64 0.323 86.1 12.24 Mith filmit form	t2.12	Triosephosphate isomerase	Gcdh ^{+/-}	892.2 ± 227.6	770.6 ± 126.5
12.14 Operate (rest) 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.24 0.05 0.07 0.00 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.01 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 0.07 <th0.07< th=""> <th0.07< th=""> 0.07</th0.07<></th0.07<>	t2.13		Gcdh ^{-/-}	834.3 ± 134.5	751.0 ± 100.7
12.13 Control 24.94 24.93 35.2 42.4 40.5 12.14 Optime (rest) 0.00 0.01 0.01 0.01 12.14 Popping/presize mutase Coft γ^{-1} 0.02 35.3 0.01.4 40.3 12.15 Coft γ^{-1} 0.02 0.02 0.01 0.01 0.01 12.21 Enolase Coft γ^{-1} 0.02 0.03 0.03 0.01 12.22 Enolase Coft γ^{-1} 0.03 0.050 0.071 1.03 0.0450 12.23 Enolase Coft γ^{-1} 0.034 0.059 0.0450 0.057 12.24 Pyruste kinase Coft γ^{-1} 2.93 ± 54.5 0.575 ± 167.3 0.057 12.24 Pyruste kinase Coft γ^{-1} 118.9 ± 22.4 44.19 ± 77.9 0.057 12.24 Pyruste kinase Coft γ^{-1} 118.9 ± 22.4 44.19 ± 77.9 0.057 12.24 Pyruste kinase Coft γ^{-1} 0.944 0.057 0.051 0.057 12.24 Pyruste kinase Coft γ^{-1} 118.2	t2.14	Characteristic 2 should be descent	p-value (t-test)	0.254	0.534
12.10Cdun140A ± 1.2240.5 ± 3.5.40.5 ± 3.5.12.11Phosphoglycerate mutaseGah ^{+1,-1} 20.752.6.320.7412.12Gah ^{+1,-1} 0.752.6.30.743.2.012.12EnolseGah ^{+1,-1} 0.750.753.763.7612.12Gah ^{+1,-1} 0.7551.857.9.358.51.78.312.12Gah ^{+1,-1} 0.5900.4500.45012.12Pyrluc (kinse0.64h ⁻¹ 0.5900.45012.24Pyrluct kinaseGah ^{+1,-1} 12.523.73.31.16.312.25Lova dfinity formGah ^{+1,-1} 12.523.75.60.65712.24Pyrluct kinaseGah ^{+1,-1} 12.523.75.60.67.712.25Lata delydiogenaseGah ^{+1,-1} 12.523.610.61.812.24AdvalueGah ^{+1,-1} 12.523.610.61.812.24AdvalueGah ^{+1,-1} 12.523.610.61.812.24Cad ovel12.520.01.80.23.78.6112.24Cad ovel12.520.13.80.13.50.61.812.34Cad ovel12.540.13.70.61.80.61.812.34Cad ovel13.716.54.31.512.34Cad ovel13.716.54.31.512.34Cad ovel13.71.61.53.412.34Cad h ^{-1,-1} 0.13.10.350.61.612.34Cad h ^{-1,-1} 0	t2.15	Giyceraidenyde 3-phosphate denydrogenase	Gcan ''	294.9 ± 153.3	38.2 ± 2.6
1.1.1 Phosphoglycrate mutate Phosphoglycrate mutate Phosphoglycrate mutate 0010 123 Phosphoglycrate mutate Gdh γ^- 17.5 ± 26.3 207.6 ± 32.0 123 Enolse Ordh γ^- 533.8 ± 79.3 553.3 ± 126.3 123 Enolse Gdh γ^- 533.8 ± 79.3 553.3 ± 126.3 123 Enolse Gdh γ^- 533.8 ± 79.3 556.3 ± 113.2 124 Enolse Gdh γ^- 533.8 ± 79.3 556.3 ± 113.2 124 Pyrtwate kinase Gdh γ^- 373.8 ± 106.3 83.4 ± 186.1 1257 Pyrtwate kinase Gdh γ^- 373.8 ± 104.3 83.4 ± 186.1 1224 Pyrtwate kinase Gdh γ^- 1262.4 ± 33.6 469.8 ± 83.1 1235 High affinity form Gdh γ^- 118.8 ± 22.4 41.9 ± 77.9 1234 Lexte dehydrogenase Gdh γ^- 91.8 ± 10.2 62.3 γ ± 66.1 1241 Cath γ^- 91.8 ± 15.5 17.3 ± 5.3 16.5 ± 4.3 1243 Table 23. TCA cycle Table 25. Cdh γ^-	t2.16		GCUN '	140.4 ± 72.2	40.5 ± 4.3
1.1 100 ph/log/refail induse 0 dri 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5 21.5	t2.17	Dhosphoglycorate mutace	$C_{cdh}^{+/-}$	0.001	0.0660
1	+2.10	riospilogiycerate mutase	Ccdh ^{-/-}	242.2 ± 33.3 2175 ± 26.3	211.4 ± 40.3 207.6 \pm 32.0
Incluse Cold γ^{-1} Civity S18 3 + 78.3 S38.3 + 128.3 223 $Call \gamma^{-1}$ S13 1 ± 115.2 S12 ± 113.2 234 P_V wate kinase $Call \gamma^{-1}$ 29.3 ± 54.0 79.3 ± 101.3 235 Dw affinity form $Call \gamma^{-1}$ 29.3 ± 54.0 79.3 ± 107.3 236 Dw affinity form $Call \gamma^{-1}$ 29.3 ± 54.0 79.3 ± 107.3 237 Dw affinity form $Call \gamma^{-1}$ 29.3 ± 54.0 79.3 ± 107.3 238 Dw affinity form $Call \gamma^{-1}$ 18.9 ± 22.4 41.9 ± 77.9 238 $Atta e lehydrogenase Call \gamma^{-1} 18.4 ± 20.4 44.9 ± 77.9 231 Lattate dehydrogenase Call \gamma^{-1} 76.1 ± 133.6 618.5 ± 90.7 233 Lattate dehydrogenase Call \gamma^{-1} 76.1 ± 133.6 0.839 233 Table 28. TCA cycle Call \gamma^{-1} 82.4 ± 15.5 17.3 ± 5.3 234 Call \gamma^{-1} 82.4 ± 15.5 17.3 ± 5.3 15.4 ± 4.3 235 Table 28. TCA cycle Call \gamma^{-1} 12.2 0.08 0.38 ± 0.21 244 Call \gamma^{-1} 12.4$	+2.13		n-Value (t-test)	0.005	0 701
$ \begin{array}{cccc} cccc} ccccccccccccccccccccccccc$	t2.20	Enolase	$Gcdh^{+/-}$	518.3 + 79.3	536.3 ± 126.3
2.23prvate kinaseprvalue (rest)0.970.46602.24prvate kinaseGath **373.3 ± 103.383.3 ± 163.12.28prvate kinaseGath **29.3 ± 54.695.3 ± 167.32.29prvate kinaseGath **18.9 ± 23.444.9 ± 77.92.20High affning formGath **18.9 ± 23.444.9 ± 77.92.31Lacta delydrogenaseGath **918.3 ± 301.262.7 ± 63.12.32Lacta delydrogenaseGath **918.3 ± 301.263.7 ± 63.12.33Table 2.8. TCA cycleGath **918.3 ± 301.263.7 ± 63.12.34Cath **91.4 ± 15.517.3 ± 5.363.5 ± 4.32.34Cath **91.4 ± 15.517.3 ± 5.363.5 ± 4.32.34Cath **92.9 ± 19.316.5 ± 4.397.4 ± 0.552.34Cath **1.0 ± 0.080.00000.000002.34Cath **1.0 ± 0.080.38 ± 0.210.34 ± 0.212.34AconitaseGath **1.0 ± 0.080.38 ± 0.212.34Cath **0.0000.00000.00000.00002.34Prvalue (rest)0.0000.00000.00002.34Cath **1.1 ± 2.63.4 ± 2.83.4 ± 2.82.44Cath **1.1 ± 2.63.4 ± 2.83.4 ± 2.82.45Gath **1.1 ± 2.63.3 ± 6.73.3 ± 6.72.44Cath **9.9 ± 0.43.9 ± 7.43.1 ± 2.63.1 ± 2.62.45Cath **9.9 ± 0.43.9 ± 7.4	t2.22		$Gcdh^{-/-}$	533.1 + 115.2	561.2 ± 113.2
224Pyruxte kinaseCafh - '-293 ± 5468343 ± 1861227Pyruxte kinaseGafh - '-293 ± 5467553 ± 1673228Pyruxte kinaseGafh - '-1262 ± 336669 ± 83.1230High affnity formGafh - '-1158 ± 22.44119 ± 77.9231Ordh - '-1358 ± 20.2633.7 ± 86.1635232Pyruxte kinaseGafh - '-1983 ± 30.12633.7 ± 86.1233Latte dehydrogenaseGafh - '-7661 ± 133.6618.6 ± 90.7234Carch e (rest)0.0180.839235Table 28. TCA cycleGafh - '-974lue (rest)0.137236Gafh - '-10.2 ± 10.316.5 ± 4.3237Girate synthaseGafh - '-10.2 ± 0.680.38 ± 0.21238AconitaseGafh - '-10.2 ± 0.680.38 ± 0.21249AconitaseGafh - '-10.4 ± 0.680.38 ± 0.21244AconitaseGafh - '-11.8 ± 2.53.4 ± 2.8245Acongenase complexGafh - '-11.8 ± 2.63.0 ± 6.6246Gafh - '-11.8 ± 2.63.0 ± 6.610.7247Garh - '-11.8 ± 2.53.4 ± 2.81.4 ± 7.7248Gafh - '-11.8 ± 2.63.0 ± 6.61.7 ± 7.3249AconitaseGafh - '-11.8 ± 2.63.0 ± 6.6244Gafh - '-11.8 ± 2.63.0 ± 6.61.7 ± 7.3245Gafh - '-11.8 ± 2.63.0 ± 6.61.7 ± 6.3246Ga	t2.23		p-Value (<i>t</i> -test)	0.599	0.4560
2.22 iow affinity form Cdh ^{2-/-} 293 ± 54.6 795.3 ± 173.3 2.23 High affinity form Cdh ^{2-/-} 152.2 ± 33.6 469.8 ± 33.1 2.24 High affinity form Cdh ^{2-/-} 158.9 ± 22.4 414.9 ± 77.9 2.31 Latte dehydrogenase Cdh ^{2-/-} 918.3 ± 301.2 632.7 ± 85.1 2.33 Cdh ^{2-/-} 918.3 ± 301.2 632.7 ± 85.1 634.6 ± 90.7 2.33 Cdh ^{2-/-} 918.3 ± 301.2 635.6 ± 90.7 2.34 Cdh ^{2-/-} 918.3 ± 301.2 635.6 ± 90.7 2.34 Cdh ^{2-/-} 918.3 ± 301.2 635.6 ± 90.7 2.34 Cdh ^{2-/-} 92.9 ± 19.3 17.3 ± 5.3 2.35 Cdh ^{2-/-} 92.9 ± 19.3 035 2.34 Aconitase Cdh ^{2-/-} 2.49 ± 13.4 0.97 ± 0.65 2.34 Aconitase Cdh ^{2-/-} 2.49 ± 1.34 0.37 ± 0.3 2.34 Aconitase Cdh ^{2-/-} 7.9 ± 0.6 0.38 ± 0.17 2.34 Conglutarate dehydrogenase complex Gdh ^{2-/-} 9.7 ± 1.6	t2.24	Pyruvate kinase	Gcdh ^{+/-}	379.3 ± 101.3	834.3 ± 186.1
12.27 P-Value (L-rest) 0.004 0.507 12.28 Pyrwate kinase Cdh ^{1-/-} 118.9 ± 23.36. 469.8 ± 83.1 12.30 High affinity form Cdh ^{-/-} 118.9 ± 20.4. 41.9 ± 77.9. 12.31 Lactate dehydrogenase Cdh ^{/-/-} 76.1. 76.3. 618.6 ± 90.7. 12.33 Cathate dehydrogenase Cdh ^{/-/-} 76.1. 76.3. 618.6 ± 90.7. 12.34 Palue 28. TCA cycle - - 0.018 0.839 12.35 Table 28. TCA cycle - - 1.3.5.5. 1.5.5. 1.3.5.5. 12.39 Carchitase Cdh ^{/-/-} 85.4 ± 15.5 1.3.5.5. 1.5.5. 1.3.5.5. 12.34 Carchitase Cdh ^{/-/-} 1.0.2.0. 0.335 0.3.5. 12.34 Carchitase Cdh ^{/-/-} 1.0.2.0. 0.3.3.5. 0.3.3.5. 12.34 Carchitase Cdh ^{/-/-} 1.0.2.0. 0.3.3.5. 0.3.3.5. 12.44 Carchitase Cdh ^{/-/-} 1.2.9.0.6. 0.3.8.5.0.	t2.25	Low affinity form	Gcdh ^{-/-}	298.3 ± 54.6	795.3 ± 167.3
2228Pyruvate kinaseCadh ^{+/-} 1262 ± 33.649.8 ± 1.1238Haffnity formCadh ^{-/-} 18.8 ± 22.441.1 ± 77.9231P-Value (t-test)0.3440.2150232Lacta de divydrogenaseCadh ^{+/-} 98.8 ± 30.1262.3.7 ± 86.1233Cadh ^{-/-} 98.8 ± 30.1262.3.7 ± 86.163.8 ± 90.7234P-Value (t-test)0.0180.83.90.83235Table 2B. TCA cycleCadh ^{+/-} 85.4 ± 15.517.3 ± 5.3238Cadh ^{+/-} 92.9 ± 19.316.5 ± 4.30.97 ± 0.65239Cadh ^{+/-} 0.1070.370.35 ± 0.352434AconiaseCadh ^{+/-} 1.02 ± 0.680.38 ± 0.21244Cadh ^{+/-} 1.02 ± 0.680.38 ± 0.21244Cadh ^{+/-} 1.02 ± 0.680.38 ± 0.21244Cadh ^{+/-} 7.7 ± 1.67.5 ± 3.8244Cadh ^{+/-} 7.7 ± 1.67.5 ± 3.8244Cadh ^{+/-} 1.17 ± 3.32.28 ± 5.4244Cadh ^{+/-} 9.49 ± 0.460.000245P-Value (t-test)0.390.912246P-Value (t-test)0.390.912247Cadh ^{+/-} 9.44 ± 2.51.30 ± 4.6248Cadh ^{+/-} 9.94 ± 2.51.30 ± 4.6244Cadh ^{+/-} 9.94 ± 2.51.30 ± 4.6245Cadh ^{+/-} 9.94 ± 2.51.30 ± 4.6246Cadh ^{+/-} 9.94 ± 2.51.30 ± 4.5247Cadh ^{+/-} 9.94 ± 2.51.5	t2.27		p-Value (t-test)	0.004	0.507
ligh affinity form Gab $^{-/-}$ 118.9 ± 22.4 41.9 ± 77.9 123 P^{Able} (-fest) 918.3 ± 30.1.2 62.3.7 ± 86.1 123 Gab $^{+/-}$ 918.3 ± 30.1.2 62.3.7 ± 86.1 123 Gab $^{+/-}$ 918.3 ± 30.1.2 61.8 ± 90.7 123 P-Value (i-test) 0.018 0.839 123 Table 28.7CA cycle	t2.28	Pyruvate kinase	Gcdh ^{+/-}	126.2 ± 33.6	469.8 ± 83.1
2.3.1 p-Value (-test) 0.344 0.2150 2.3.3 Icatate dehydrogenase Gdh ^{-/-} 766.1 ± 133.6 618.6 ± 90.7 2.3.4 Gdh ^{-/-} 766.1 ± 133.6 618.6 ± 90.7 2.3.5 Table 2B. TCA cycle 0.18 60.33 2.3.6 Gdh ^{-/-} 85.4 ± 15.5 17.3 ± 5.3 2.3.8 Gdh ^{-/-} 92.9 ± 19.3 165.5 ± 4.3 2.3.9 Gcdh ^{-/-} 92.9 ± 19.3 165.5 ± 4.3 2.3.9 P-Value (-test) 0.137 0.535 2.4.1 Aconitase Gcdh ^{-/-} 92.9 ± 19.3 165.5 ± 4.3 2.4.1 Aconitase Gcdh ^{-/-} 1.02 ± 0.68 0.38 ± 0.21 2.4.1 Aconitase Gcdh ^{-/-} 1.02 ± 0.68 0.38 ± 0.21 2.4.1 Aconitase Gcdh ^{-/-} 1.02 ± 0.68 0.000 2.4.4 Complex I Gcdh ^{-/-} 1.02 ± 0.68 0.000 2.4.4 Complex I Gcdh ^{-/-} 1.17 ± 3.3 2.8 ± 5.4 2.4.4 Gcdh ^{-/-} 1.17 ± 3.3 2.8 ± 5.4 5.4 2.4.4 Gcdh ^{-/-} 1.17 ± 3	t2.39	High affinity form	Gcdh ^{-/-}	118.9 ± 22.4	441.9 ± 77.9
12.22 Lactate dehydrogenase Gcdh γ^{-} 9183 ± 301.2 623.7 ± 86.1 12.33 Gcdh γ^{-} 9183 ± 301.2 623.7 ± 86.1 12.34 p-Value (t-test) 0.018 0.839 12.35 Table 28. TCA cycle - - 12.34 Gcdh γ^{-} 92.9 ± 19.3 16.5 ± 4.3 12.39 Gcdh γ^{-} 92.9 ± 19.3 0.5535 12.40 Aconitase Gcdh γ^{-} 10.2 ± 0.68 0.83 ± 0.21 12.41 Gcdh γ^{-} 0.2 ± 0.68 0.83 ± 0.21 0.000 0.0000 12.42 Aconitase Gcdh γ^{-} 0.2 ± 0.68 0.83 ± 0.21 0.44 ± 2.8 12.42 Gcdh γ^{-} 0.2 ± 0.68 0.67 0.000 0.0000 12.43 Conglutarate dehydrogenase complex Gcdh γ^{-} 8.1 ± 2.5 3.4 ± 2.8 12.44 Gcdh γ^{-} 8.1 ± 2.5 3.4 ± 2.8 4.4 ± 2.8 12.45 Gcdh γ^{-} 11.8 ± 2.6 23.0 ± 6.4 12.46 Gcdh γ^{-} 9.9 ± 0.4 2.5 ± 11.7 12.45 Gcmd γ^{-} 9.9 ± 0.4 2.5	t2.31		p-Value (<i>t</i> -test)	0.344	0.2150
12.33 Cech ^{+/-} 766. \pm 133.6 618.6 \pm 90.7 12.33 Table 2B. TCA cycle PValue (1-test) 0.018 0.018 12.35 Table 2B. TCA cycle 5.4 \pm 15.5 17.3 \pm 5.3 12.36 Cartar synthase Cach ^{+/-} 8.5.4 \pm 15.5 17.3 \pm 5.3 12.39 Cartar synthase Cach ^{+/-} 9.2.9 \pm 1.9.3 16.5 \pm 4.3 12.30 Aconitase Cach ^{+/-} 9.2.9 \pm 1.9.3 0.55 \pm 4.3 12.40 Aconitase Cach ^{+/-} 9.2.9 \pm 1.9.3 0.55 \pm 4.3 12.41 P-Value (1-test) 0.137 0.53 \pm 0.5 12.42 Acongutarate dehydrogenase complex Cach ^{+/-} 1.02 \pm 0.68 0.38 \pm 0.21 12.42 2-Oxoglutarate dehydrogenase complex Cach ^{+/-} 8.1 \pm 2.5 3.4 \pm 2.8 12.44 Table 2C. Respiratory chain 1.7 \pm 3.3 22.8 \pm 5.4 12.45 Complex I Cach ^{+/-} 1.1 \pm 3.3 23.9 \pm 5.4 12.46 Complex II Cach ^{+/-} 9.4 \pm 2.5 1.3 \pm 4.2 12.45 Complex II Cach ^{+/-} 9.4 \pm 6.0 2.2 \pm 5.4	t2.32	Lactate dehydrogenase	Gcdh ^{+/-}	918.3 ± 301.2	623.7 ± 86.1
22.34 p-Value (r-test) 0.018 0.83 22.35 Table 2B. TCA cycle 22.37 Circate synthase $Cdh^{+/-}$ $S2.9 \pm 19.3$ 15.5 ± 4.3 22.39 $Cdh^{-/-}$ $S2.9 \pm 19.3$ 15.5 ± 4.3 22.40 $Aconitase$ $Cdh^{-/-}$ 29 ± 19.3 0.555 22.41 $Aconitase$ $Cdh^{-/-}$ 1.02 ± 0.68 0.38 ± 0.21 22.42 $Aconitase$ $Cdh^{-/-}$ 1.02 ± 0.68 0.38 ± 0.21 22.44 $CAoguitarate dehydrogenase complex Cdh^{-/-} 1.02 \pm 0.68 0.38 \pm 0.21 22.44 CAoguitarate dehydrogenase complex Cdh^{-/-} 1.92 \pm 0.68 0.000 22.44 Cooplex II Cdh^{-/-} 1.17 \pm 3.3 22.8 \pm 5.4 24.4 Cooplex II Cdh^{+/-} 1.92 \pm 0.68 23.0 \pm 6.4 25.4 Complex II Cdh^{+/-} 9.4 \pm 2.5 13.0 \pm 4.6 25.4 Complex III Cedh^{+/-} 9.4 \pm 2.5 13.0 \pm 4.6 25.4 Complex III Cedh^{+/-} 9.4 \pm 2.5 13.2 \pm 4.7$	t2.33		Gcdh '	766.1 ± 133.6	618.6 ± 90.7
12.37 Table 2B. TCA cycle 17.3 ± 5.3 22.37 Citrate synthase Cach -'- 92.9 ± 19.3 16.5 ± 4.3 22.38 Cach -'- 92.9 ± 19.3 0.535 12.39 P'Value (r.test) 0.137 0.535 12.30 Aconitase Cach -'- 2.49 ± 1.34 0.97 ± 0.65 12.41 Aconitase Cach -'- 1.02 ± 0.68 0.38 ± 0.21 12.42 Cach -'- 1.02 ± 0.68 0.38 ± 0.21 12.42 Cach -'- 1.02 ± 0.68 0.38 ± 0.21 12.42 Cach -'- 1.02 ± 0.68 0.38 ± 0.21 12.42 Cach -'- 1.02 ± 0.68 0.38 ± 0.21 12.42 Cach -'- 8.1 ± 2.5 3.4 ± 2.8 12.44 Cach -'- 1.17 ± 3.3 2.2.8 ± 5.4 12.45 Complex I Cach -'- 1.17 ± 2.3 2.2.8 ± 5.4 12.44 Cach -'- 1.8 ± 2.5 2.3.0 ± 6.4 12.45 Complex II Cach -'- 9.9 ± 2.6 1.3.7 ± 4.7 12.46 Cach -'- 9.9 ± 2.6 1.3.7 ± 4.7 12.55 Complex II </td <td>t2.34</td> <td></td> <td>p-Value (<i>t</i>-test)</td> <td>0.018</td> <td>0.839</td>	t2.34		p-Value (<i>t</i> -test)	0.018	0.839
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	t2.35 t2.36	Table 2B. TCA cycle			
$ \begin{array}{ccccc} 1 \\ 1 \\ 1 \\ 2 \\ 2 \\ 2 \\ 2 \\ 2 \\ 2 \\ 2 \\$	t2.37	Citrate synthase	Gcdh ^{+/-}	85.4 ± 15.5	17.3 ± 5.3
2.39 Aconitase $P_V lue (t-test)$ 0.137 0.535 2.40 Aconitase $Gdh^{-/-}$ 2.49 ± 1.34 0.79 ± 0.65 2.41 $P_V lue (t-test)$ 0.000 0.000 2.42 $P_V lue (t-test)$ 0.000 0.000 2.43 $2-corgiturate dehydrogenase complex Gdh^{-/-} 8.1 \pm 2.5 3.4 \pm 2.8 2.44 Gdh^{-/-} 0.664 0.000 0.000 2.45 -P_V lue (t-test) 0.664 0.000 0.000 2.46 Complex I Cdh^{+/-} 1.7 \pm 3.3 22.8 \pm 5.4 2.47 Table 2C. Respiratory chain P_V lue (t-test) 0.99 9.12 2.48 Complex II Cdh^{+/-} 9.4 \pm 2.5 13.0 \pm 4.6 2.49 Codh^{+/-} 9.4 \pm 2.5 13.0 \pm 4.6 7.4 + 7.7 2.51 Complex III Cdh^{+/-} 9.9 \pm 2.6 13.0 \pm 4.6 2.52 Conflex^{-/-} 9.9 \pm 2.6 13.0 \pm 4.6 7.4 + 7.5 2.54 Conflex III Cdh^{+/-} 9.9 \pm 1.6 0.568 $	t2.38	•	Gcdh ^{-/-}	92.9 ± 19.3	16.5 ± 4.3
2:40 Aconitase Gcdh ^{+/-} 2:49 ± 1.34 0.97 ± 0.65 2:41 Gcdh ^{-/-} 102 ± 0.68 0.38 ± 0.21 2:42 p-Value (t-test) 0.000 0.0000 2:43 2-Oxoglutarate dehydrogenase complex Gcdh ^{-/-} 7.7 ± 1.6 7.5 ± 3.8 2:44 Gcdh ^{-/-/-} 8.1 ± 2.5 0.000 0.000 2:45 p-Value (t-test) 0.464 0.000 0.000 2:46 Table 2C. Respiratory chain 11.7 ± 3.3 22.8 ± 5.4 2:47 Table 2C. Respiratory chain 11.8 ± 2.6 3.0 ± 4.6 2:50 Complex I Gcdh ^{-/-} 9.410 (t-test) 0.939 0.912 2:51 Complex II Gcdh ^{-/-} 9.0 ± 2.6 13.7 ± 4.7 2:52 Gcdh ^{-/-} 9.0 ± 2.6 13.7 ± 4.7 2:54 Complex III Gcdh ^{-/-} 9.0 ± 2.6 0.57 2:54 Complex III Gcdh ^{-/-} 9.0 ± 2.6 13.7 ± 4.7 2:55 Gcdh ^{-/-} 9.0 ± 2.6 13.7 ± 6.7 15.7 2:54 Complex III Gcdh ^{-/-} 9.0 ± 2.6 13.7 ± 6.7 <td>t2.39</td> <td></td> <td>p-Value (<i>t</i>-test)</td> <td>0.137</td> <td>0.535</td>	t2.39		p-Value (<i>t</i> -test)	0.137	0.535
12.41 Gcdh ^{-/-} 1.02 ± 0.68 0.38 ± 0.21 12.42 p-Value (t-test) 0.000 0.0000 12.43 2-0xoglutarate dehydrogenase complex Gcdh ^{-/-} 7.7 ± 1.6 7.5 ± 3.8 12.44 Gcdh ^{-/-} 8.1 ± 2.5 3.4 ± 2.8 12.45	t2.40	Aconitase	Gcdh ^{+/-}	2.49 ± 1.34	0.97 ± 0.65
12.42 P-Value (rest) 0.000 0.0000 12.43 2-0xoglutarate dehydrogenase complex Gdh ^{1/-} 7.7 ± 1.6 for 7.5 ± 3.8 (1.2 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.8 ± 3.4 ± 2.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ± 4.4 ±	t2.41		Gcdh ^{-/-}	1.02 ± 0.68	0.38 ± 0.21
2-4xguittarate dehydrogenase complex $Gcdh^{+/-}$ 7,7 ± 1,6 7,5 ± 3,8 2:44 $Gcdh^{-/-}$ 8,1 ± 2,5 3,4 ± 2,8 2:45 p -Value (tetst) 0,464 0,000 12:46 $Table 2C. Respiratory chain Table 2C. Respiratory chain 22.8 ± 5.4 2:47 Table 2C. Respiratory chain Ccdh^{+/-} 11.7 ± 3.3 22.8 ± 5.4 2:49 Gcdh^{-/-} 11.8 ± 2.6 23.0 ± 6.4 2:50 P-Value (t-test) 0.939 0.912 2:51 Complex II Gcdh^{-/-} 9.0 ± 2.6 13.0 ± 4.6 2:52 P-Value (t-test) 0.568 0.617 2:54 Complex III Gcdh^{-/-} 39.9 ± 7.4 21.5 ± 7.5 2:55 Gcdh^{-/-} 39.9 ± 7.4 21.6 ± 7.5 51.01 2:54 Complex IV Gcdh^{-/-} 39.9 ± 2.7.1 53.5 ± 315.01 2:55 Gcdh^{-/-} 17.2.6 ± 53.0.1 53.5 ± 315.01 2:56 Gcdh^{-/-} 53.7 ± 8.9 20.7 ± 7.6 2:57 Gcdh^{-/-} 53.7 ± 8.9 20.7 ± 7.6 2:50 <$	t2.42		p-Value (<i>t</i> -test)	0.000	0.0000
12.44 $Gcdn^{-/-}$ 8.1 ± 2.5 3.4 ± 2.8 12.45 p -Value (i-test) 0.664 0.000 12.47 $Table 2C. Respiratory chain$ $Z2.8 \pm 5.4$ $Complex I$ 22.8 ± 5.4 12.49 $Gcdh^{+/-}$ 11.7 ± 3.3 22.8 ± 5.4 12.49 $Gcdh^{-/-}$ 11.8 ± 2.6 23.0 ± 6.4 12.50 $Gcdh^{+/-}$ 9.4 ± 2.5 13.0 ± 4.6 12.51 Complex II $Gcdh^{+/-}$ 9.0 ± 2.6 13.7 ± 4.7 12.53 $Gcdh^{+/-}$ 9.0 ± 2.6 0.617 13.7 ± 4.7 12.54 Complex III $Gcdh^{+/-}$ 9.0 ± 2.6 0.617 12.6 ± 7.5 12.54 Complex III $Gcdh^{-/-}$ 39.9 ± 7.4 21.6 ± 7.5 0.736 12.55 Complex IV $Gcdh^{-/-}$ 1123.9 ± 227.1 57.96 ± 176.0 0.955 12.54 ATP synthase $Gcdh^{-/-}$ 123.9 ± 227.1 57.96 ± 176.0 $0.79.7 \pm 6.8$ 12.55 $Gcdh^{-/-}$ 123.9 ± 27.1 57.96 ± 176.0 $0.79.7 \pm 6.8$ $0.79.7 \pm 6.8$ $0.79.7 \pm 6.8$ $0.79.7 \pm 6.8$	t2.43	2-Oxoglutarate dehydrogenase complex	Gcdh ^{+/-}	7.7 ± 1.6	7.5 ± 3.8
12.45 p-Value (r.test) 0.464 0.000 12.47 Table 2C. Respiratory chain 11.7 ± 3.3 22.8 ± 5.4 12.48 Complex I Gcdh ^{-/-} 11.8 ± 2.6 23.0 ± 6.4 12.49 p-Value (r.test) 0.939 0.912 12.50 p-Value (r.test) 0.939 0.912 12.51 Complex II Gcdh ^{-/-} 9.0 ± 2.6 13.0 ± 4.6 12.52 Gcdh ^{-/-} 9.0 ± 2.6 13.7 ± 4.7 12.53 Complex III Gcdh ^{-/-} 9.0 ± 2.6 13.7 ± 4.7 12.54 Complex III Gcdh ^{-/-} 9.0 ± 2.6 13.7 ± 4.7 12.55 Gcdh ^{-/-} 9.9 ± 7.4 21.6 ± 7.5 12.54 Complex III Gcdh ^{-/-} 39.9 ± 7.4 21.6 ± 7.5 12.55 Gcdh ^{-/-} 1123.9 ± 227.1 579.6 ± 176.0 12.58 Gcdh ^{-/-} 1123.9 ± 227.1 579.6 ± 176.0 12.59 P-Value (r.test) 0.654 0.955 12.60 Gcdh ^{-/-} 53.7 ± 8.9 0.7 ± 7.6 12.61 Gcdh ^{-/-} 19.7 ± 6.8 12.4 ± 53.2	t2.44		Gcdh ^{-/-}	8.1 ± 2.5	3.4 ± 2.8
1247 Table 2C. Respiratory chain 22.8 ± 5.4 12.48 Complex I 11.7 ± 3.2 22.8 ± 5.4 12.49 $Gcdh^{-/-}$ 11.8 ± 2.6 23.0 ± 6.4 12.50 p -Value (r-test) 0.939 0.912 12.51 Complex II $Ccdh^{+/-}$ 9.4 ± 2.5 13.0 ± 4.6 12.52 Complex II $Ccdh^{+/-}$ 9.0 ± 2.6 13.7 ± 4.7 12.53 Omplex III $Ccdh^{+/-}$ 39.4 ± 6.0 22.5 ± 11.7 12.54 Complex III $Ccdh^{+/-}$ 39.9 ± 7.4 21.6 ± 7.5 12.56 Omplex IV $Ccdh^{+/-}$ 39.9 ± 7.4 21.6 ± 7.5 12.57 Complex IV $Ccdh^{-/-}$ 1172.6 ± 530.1 583.5 ± 315.0 12.58 p -Value (r-test) 0.797 0.736 12.59 p -Value (t-test) 0.654 0.955 12.60 ATP synthase $Ccdh^{-/-}$ 51.24 ± 50.9 12.4 ± 53.2 12.61 p -Value (t-test) 0.701 0.635 12.4 ± 53.2 12.64 $Ccdh^{-/-}$ $31.9 \pm 7.$	t2.45		p-Value (<i>t</i> -test)	0.464	0.000
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	t2.46 t2.47	Table 2C. Respiratory chain			
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	t2.48	Complex I	Gcdh ^{+/-}	11.7 ± 3.3	22.8 ± 5.4
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	t2.49		Gcdh ^{-/-}	11.8 ± 2.6	23.0 ± 6.4
t2.51 Complex II $Gcdh^{+/-}$ 9.4 ± 2.5 13.0 ± 4.6 t2.52 $Gcdh^{-/-}$ 9.0 ± 2.6 13.7 ± 4.7 t2.53 $PValue (t-test)$ 0.568 0.617 t2.54 Complex III $Gcdh^{+/-}$ 39.4 ± 6.0 22.5 ± 11.7 t2.55 $Gcdh^{+/-}$ 39.9 ± 7.4 21.6 ± 7.5 t2.56 $P-Value (t-test)$ 0.797 0.736 t2.57 Complex IV $Gcdh^{+/-}$ 1172.6 ± 530.1 58.35 ± 315.0 t2.58 $Gcdh^{-/-}$ 1123.9 ± 227.1 579.6 ± 176.0 t2.59 $P-Value (t-test)$ 0.654 0.955 t2.60 ATP synthase $Gcdh^{-/-}$ 54.9 ± 13.2 19.7 ± 6.8 t2.61 $P-Value (t-test)$ 0.654 0.955 t2.62 $P-Value (t-test)$ 0.635 20.7 ± 7.6 t2.62 $P-Value (t-test)$ 0.701 0.635 t2.63 Flux complex I-III $Gcdh^{-/-}$ 31.4 ± 6.3 121.4 ± 53.2 t2.64 $Gcdh^{-/-}$ 6.6 ± 0.8 11.7 ± 6.4 t2.65 <	t2.50		p-Value (<i>t</i> -test)	0.939	0.912
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	t2.51	Complex II	Gcdh ^{+/-}	9.4 ± 2.5	13.0 ± 4.6
$t2.53$ p-Value (<i>t</i> -test) 0.568 0.617 $t2.54$ Complex III $Gcdh^{+/-}$ 39.4 ± 6.0 22.5 ± 11.7 $t2.56$ p-Value (<i>t</i> -test) 39.9 ± 7.4 21.6 ± 7.5 $t2.56$ p-Value (<i>t</i> -test) 0.797 0.736 $t2.57$ Complex IV $Gcdh^{+/-}$ 1172.6 ± 530.1 583.5 ± 315.0 $t2.58$ $Gcdh^{-/-}$ 1123.9 ± 227.1 579.6 ± 176.0 $t2.59$ p-Value (<i>t</i> -test) 0.654 0.955 $t2.60$ ATP synthase $Gcdh^{+/-}$ 54.9 ± 13.2 19.7 ± 6.8 $t2.61$ $P-Value (t-test)$ 0.701 0.655 $t2.64$ $P-Value (t-test)$ 0.701 0.655 $t2.64$ $P-Value (t-test)$ 0.701 0.635 $t2.64$ $P-Value (t-test)$ 0.815 0.453 $t2.64$ $P-Value (t-test)$ 0.815 0.453 $t2.65$ $P-Value (t-test)$ 0.815 0.453 $t2.64$ $P-Value (t-test)$ 0.985 0.838	t2.52		Gcdh ^{-/-}	9.0 ± 2.6	13.7 ± 4.7
$t_{2.54}$ Complex III $Ccdh^{-/-}$ 39.4 ± 6.0 22.5 ± 11.7 $t_{2.55}$ $Ccdh^{-/-}$ 39.9 ± 7.4 21.6 ± 7.5 $t_{2.57}$ Complex IV $Ccdh^{+/-}$ 0.797 0.736 $t_{2.57}$ Complex IV $Ccdh^{+/-}$ 1172.6 ± 530.1 583.5 ± 315.0 $t_{2.58}$ $Ccdh^{+/-}$ 1123.9 ± 227.1 579.6 ± 176.0 $t_{2.59}$ p -Value (t-test) 0.654 0.955 $t_{2.60}$ ATP synthase $Ccdh^{+/-}$ 54.9 ± 13.2 19.7 ± 6.8 $t_{2.61}$ p -Value (t-test) 0.654 0.955 $t_{2.61}$ p -Value (t-test) 0.701 0.635 $t_{2.64}$ p -Value (t-test) 0.701 0.635 $t_{2.64}$ p -Value (t-test) 0.815 0.453 $t_{2.64}$ p -Value (t-test) 0.815 0.453 $t_{2.65}$ p -Value (t-test) 0.985 0.838	t2.53		p-Value (<i>t</i> -test)	0.568	0.617
$t^{2.55}$ $Ccdh^{-/}$ 39.9 ± 7.4 21.6 ± 7.5 $t^{2.56}$ $p^{-Value(t-test)}$ 0.797 0.736 $t^{2.57}$ Complex IV $Gcdh^{+/-}$ 1172.6 ± 530.1 58.5 ± 315.0 $t^{2.58}$ $Gcdh^{-/-}$ 1123.9 ± 227.1 579.6 ± 176.0 $t^{2.59}$ $p^{-Value(t-test)}$ 0.654 0.955 $t^{2.60}$ ATP synthase $Gcdh^{+/-}$ 54.9 ± 13.2 19.7 ± 6.8 $t^{2.61}$ $p^{-Value(t-test)}$ 0.701 0.635 $t^{2.64}$ $p^{-Value(t-test)}$ 0.701 0.635 $t^{2.64}$ $Gcdh^{-/-}$ 31.9 ± 7.5 132.4 ± 50.9 $t^{2.64}$ $p^{-Value(t-test)}$ 0.815 0.453 $t^{2.65}$ $p^{-Value(t-test)}$ 0.815 0.453 $t^{2.66}$ $flux$ complex II-III $Gcdh^{+/-}$ 2.6 ± 0.8 11.7 ± 6.4 $t^{2.68}$ $p^{-Value(t-test)}$ 0.985 0.838 0.838	t2.54	Complex III	Gcdh ''	39.4 ± 6.0	22.5 ± 11.7
$L_{2.67}$ Complex IV $Gcdh^{+/-}$ 1172.6 ± 530.1 583.5 ± 315.0 $L_{2.58}$ $Gcdh^{-/-}$ 1123.9 ± 227.1 579.6 ± 176.0 $L_{2.59}$ p -Value (<i>t</i> -test) 0.654 0.955 $L_{2.61}$ p -Value (<i>t</i> -test) 0.654 0.955 $L_{2.61}$ $Gcdh^{+/-}$ 53.7 ± 8.9 20.7 ± 7.6 $L_{2.62}$ p -Value (<i>t</i> -test) 0.701 0.635 $L_{2.64}$ $Gcdh^{+/-}$ 31.9 ± 7.5 32.4 ± 50.9 $L_{2.65}$ p -Value (<i>t</i> -test) 0.815 0.453 $L_{2.65}$ p -Value (<i>t</i> -test) 0.815 0.453 $L_{2.66}$ Flux complex II-III $Gcdh^{+/-}$ 2.6 ± 0.8 11.7 ± 6.4 $L_{2.67}$ $Gcdh^{-/-}$ 2.6 ± 1.3 11.5 ± 3.0 $L_{2.68}$ p -Value (<i>t</i> -test) 0.985 0.838	t2.55		GCUN '	39.9 ± 7.4	21.0 ± 7.5
L2.57 Complex IV 1172.5 \pm 35.1 535.1 \pm 31.30 L2.58 Gcdh ^{-/-} 1122.9 \pm 227.1 579.6 \pm 176.0 L2.59 p-Value (t-test) 0.654 0.955 L2.60 ATP synthase Gcdh ^{-/-} 54.9 \pm 13.2 19.7 \pm 6.8 L2.61 Gcdh ^{-/-} 53.7 \pm 8.9 20.7 \pm 7.6 L2.62 p-Value (t-test) 0.701 0.635 L2.63 Flux complex I-III Gcdh ^{-/-} 31.9 \pm 7.5 32.4 \pm 50.9 L2.64 Gcdh ^{-/-} 31.4 \pm 6.3 121.4 \pm 53.2 L2.65 p-Value (t-test) 0.815 0.453 L2.66 Flux complex II-III Gcdh ^{+/-} 2.6 \pm 0.8 11.7 \pm 6.4 L2.67 Gcdh ^{-/-} 2.6 \pm 1.3 11.5 \pm 3.0 L2.68 p-Value (t-test) 0.985 0.838	t2.50	Complex IV	p-value (<i>i</i> -test)	0.797	0./30 5925 2150
$L2.69$ $p-Value (t-test)$ 0.654 0.955 $L2.69$ ATP synthase $Gcdh^{+/-}$ 54.9 ± 13.2 19.7 ± 6.8 $L2.61$ $Gcdh^{-/-}$ 53.7 ± 8.9 20.7 ± 7.6 $L2.62$ $p-Value (t-test)$ 0.701 0.635 $L2.63$ Flux complex I–III $Gcdh^{+/-}$ 31.9 ± 7.5 132.4 ± 50.9 $L2.64$ $Gcdh^{-/-}$ 31.4 ± 6.3 121.4 ± 53.2 $L2.65$ $p-Value (t-test)$ 0.815 0.453 $L2.66$ Flux complex II–III $Gcdh^{+/-}$ 2.6 ± 0.8 11.7 ± 6.4 $L2.66$ Flux complex II–III $Gcdh^{-/-}$ 2.6 ± 1.3 11.5 ± 3.0 $L2.68$ $p-Value (t-test)$ 0.985 0.838	12.07	complex iv	$Ccdh^{-/-}$	1172.0 ± 330.1 1123.9 ± 227.1	535.5 ± 515.0 5796 + 1760
2.60 ATP synthase $Gcdh^{+/-}$ 54.9 ± 13.2 19.7 ± 6.8 2.61 $Gcdh^{-/-}$ 53.7 ± 8.9 20.7 ± 7.6 2.62 p -Value (t-test) 0.701 0.635 2.63 Flux complex I–III $Gcdh^{+/-}$ 31.9 ± 7.5 132.4 ± 50.9 2.64 $Gcdh^{-/-}$ 31.4 ± 6.3 121.4 ± 53.2 2.65 p -Value (t-test) 0.815 0.453 2.66 Flux complex II–III $Gcdh^{+/-}$ 2.6 ± 0.8 11.7 ± 6.4 2.67 $Gcdh^{-/-}$ 2.6 ± 1.3 11.5 ± 3.0 2.68 p -Value (t-test) 0.985 0.838	+2.50		n-Value (t-test)	0.654	0.955
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	t2.60	ATP synthase	$Gcdh^{+/-}$	549 + 132	197 ± 68
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	t2.61		$Gcdh^{-/-}$	53.7 ± 8.9	20.7 ± 7.6
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	t2.62		p-Value (<i>t</i> -test)	0.701	0.635
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	t2.63	Flux complex I–III	Gcdh ^{+/-}	31.9 ± 7.5	132.4 ± 50.9
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	t2.64		$Gcdh^{-/-}$	31.4 ± 6.3	121.4 ± 53.2
$\begin{array}{ccccccc} t2.66 & Flux complex II-III & & & & & & & & & & & & & & & & $	t2.65		p-Value (<i>t</i> -test)	0.815	0.453
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	t2.66	Flux complex II–III	Gcdh ^{+/-}	2.6 ± 0.8	11.7 ± 6.4
t2.68 p-Value (<i>t</i> -test) 0.985 0.838	t2.67		Gcdh ^{-/-}	2.6 ± 1.3	11.5 ± 3.0
	t2.68		p-Value (<i>t</i> -test)	0.985	0.838

in these mice. This may be associated with an increase in neurotoxicdicarboxylic metabolites that induce the acute clinical phenotype.

496 4.3. Threshold for neurotoxicity

For GA-I patients, there is no known correlation between the genotype and clinical phenotype or the biochemical and the clinical phenotypes, whereas the genotype correlates with the biochemical
phenotype [7,29,17]. Furthermore, there is a high variability in the

severity of the disease course, even in siblings and in other patients 501 with the same *GCDH* gene mutations [17]. Considering the toxic metab-502 olite and trapping hypotheses it is tempting to speculate that the vari-503 ability of the natural history of GA-I patients might be based on 504 different intracerebral concentrations of neurotoxic metabolites and 505 that such differences might be influenced by the genetic background 506 and environmental factors. 507

We therefore wondered whether symptomatic and asymptomatic 508 $Gcdh^{-/-}$ mice differed in their GA and 3-OH-GA concentrations during 509

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Fig. 5. Brain pathology of *Gcdh*^{-/-} mice. Histological analysis of hippocampal sections of (A) WT control, (B) +/- 376 mg/d Lys, (C) -/- 85 mg/d Lys, (D) -/- 235 mg/d Lys, (E) -/- 280 mg/d Lys and (F) -/- 376 mg/d Lys mice are depicted (for each condition HE (left) and Klüver–Barrera (right) stainings are depicted). In comparison to WT control and +/- mice, *Gcdh*^{-/-} mice display CNS spongiosis which is most prominent in the hippocampus (scale bar = 500 µm for all images). (G) Box-plots of different genetic and dietary conditions are depicted. Statistical analysis was performed using non-parametric Wilcoxon test. A significance level of alpha = 0.05 was selected for all tests (* = p < 0.05). (+/- 376 mg/g Lys: n = 4; -/- 85 mg/d Lys: n = 4; -/- 235 mg/d Lys: n = 5; -/- 280 mg/d: n = 3; -/- 376 mg/d: n = 3).

L-lysine exposure. In fact, symptomatic mice showed a much higher
 increase in cerebral and hepatic GA and – less pronounced – in cerebral
 3-OH-GA concentrations than asymptomatic mice.

513 4.4. Oral L-lysine loading induced pathology results in enhanced catabolism

⁵¹⁴ Due to reduced food intake oral L-lysine loading leads to a catabolic ⁵¹⁵ state of symptomatic animals and, thereby, increases the breakdown ⁵¹⁶ of L-lysine and other amino acids. Symptomatic $Gcdh^{-/-}$ mice showed ⁵¹⁷ weight loss upon start of L-lysine exposure as well as increased produc-⁵¹⁸ tion of urea secondary to increased ammonia production. Normal serum ⁵¹⁹ cystatin C and creatinine concentrations virtually exclude renal insuffi-⁵²⁰ ciency as cause of elevated urea concentrations upon treatment.

Zinnanti and colleagues put forward the idea that a high L-lysine diet
 diminishes 2-oxoglutarate levels due to increased saccharopine path way activity [46]. However, in our study we did not find evidence for
 decreased L-glutamate or L-glutamine levels as a consequence of 2 oxoglutarate depletion. Our data also do not support the suggestion
 that hyperammonemia plays a pathomechanistic role [54] since we

did not find increased cerebral L-glutamine or ammonia concentrations. 527 In analogy, hyperammonemic episodes have never been reported for 528 patients with GA-I. 529

4.5. L-lysine diet induced changes of energy metabolism

530

Evaluation of energy metabolism in symptomatic and asymptomatic 531 mice identified functional impairment and secondary changes of en-532 zyme activities in glycolysis and TCA cycle, whereas activities of respira-533 tory chain complexes remained unchanged. Most notably, activities of 534 GAPDH and aconitase activities were markedly decreased in the brain 535 of $Gcdh^{-/-}$ mice after L-lysine exposure. No increase of citrate synthase 536 activity was found in $Gcdh^{-/-}$ mice upon L-lysine exposure suggesting that mitochondrial proliferation is not induced to compensate for the 538 enzymatic assays record steady state activity and do not reflect inhibito-540 ry effects of accumulating metabolites. In a previous study we have 541 shown that GA, 3-OH-GA, or glutaryl-CoA do not have a direct inhibitor 542 effect on proteins of energy metabolism except for an inhibition of 543 OGDHc by glutaryl-CoA [22]. Therefore, the observed changes in our 544

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study are most likely due to metabolic stress induced by increased L-lysine intake.

547 GAPDH, OGDHc, and aconitase have shown to be highly vulnerable
548 for oxidative stress decreasing their catalytic activity [60–63]. In concert
549 with the direct inhibition of OGDHc by glutaryl-CoA these findings indi550 cate that impairment of energy homeostasis plays an important role in
551 neuropathogenesis of GA-I.

 $_{552}$ 4.6. Hippocampal spongiosis is a major neuropathological finding in Gcdh^{-/} $_{553}$ — mice but does not depend on dietary L-lysine supply

Histological evaluation of $Gcdh^{-/-}$ mice following L-lysine exposure 554revealed no considerable pathology of the striatum. In contrast, 555556 $Gcdh^{-/-}$ mouse brains showed increased spongiosis, most prominently within the hippocampus. Although hippocampal spongiosis was signif-557icantly increased in $Gcdh^{-/-}$ mouse brain as compared to $Gcdh^{+/-}$ 558 mice, no considerable differences were detected between symptomatic 559 and asymptomatic mice. Further, neuropathological changes were inde-560pendent of the amount of L-lysine supplied. This is in contrast to a pre-561vious study which suggested that the high L-lysine diet induces a striatal 562pathology similar to that of GA-I patients [49]. 563

Hippocampal neurons are highly vulnerable to various toxins and 564565ROS and thus are involved in many neurodegenerative diseases [64]. 566 An in vitro study has demonstrated that hippocampal rat neurons are susceptible to 3-OH-GA and ROS formation [55]. Chronic damage of 567these neurons may occur in $Gcdh^{-/-}$ mice due to long-term exposure 568 to high cerebral GA and 3-OH-GA concentration during postnatal and 569570maybe also prenatal brain development. Selective spongiosis in hippocampus and cortex has been found in neurodegenerative diseases 571such as Alzheimer's disease [65-67]. PAS staining indicates that 572spongiosis occurs due to loss of neuronal cells as it has been suggested 573574before by Zinnanti et al. [46] and been demonstrated in asymptomatic $Gcdh^{-/-}$ mice on a standard diet [47]. In GA-I patients, however, no ev-Q8 576idence of a significant hippocampal pathology has been found yet [29]. This may reflect species-dependent differences 577

578 4.7. Gcdh^{-/-} mice as an animal model for GA-I

The acute clinical phenotype in $Gcdh^{-/-}$ mice induced by a high L-579lysine depends on various parameters such as the age at start of treat-580ment, gender, genetic modifiers, and the amount of oral L-lysine supply. 581 These parameters, as far as they are known, need to be carefully con-582trolled to achieve a reliable and reproducible murine model for GA-I. Al-583though this model resembles the biochemical phenotype of GA-I 584patients with a high excreting phenotype, neuropathological changes 585586were significantly discrepant to the human phenotype. Specifically, 587major changes were observed in the murine hippocampus, whereas the striatum remained virtually unaffected. In addition, the same hippo-588campal changes were observed in $Gcdh^{-/-}$ mice both on a standard diet 589or on a high L-lysine diet and thus are likely induced by chronic rather 590than acute neurotoxicity. Since both, the spontaneously developing 591592and the high L-lysine diet-induced clinical phenotype, differ from that 593of GA-I patients, clinical endpoints for therapeutic studies on Gcdh⁻ mice should be carefully chosen. Of note, the lack of apparent neuropa-594595thology in the sensitive mice is suggestive of toxicity that affects only a 596relatively minor population of neurons. In contrast to the global injury 597reported by Zinnanti and others, this is more consistent with the observed phenotype in patients with GA-I. Future studies have to identify 598 the subpopulation of affected neurons which will further help to evalu-599 ate the suitability of this animal model. 600

For the first time, we have demonstrated that the genetic background and gender plays an important role in the clinical phenotype. This needs to be addressed more specifically in future studies in order to elucidate factors that influence cerebral L-lysine oxidation and the susceptibility to neurotoxic metabolites and thus might be used for the development of new therapeutic strategies.

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