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Slc22a5 haploinsufficiency does not aggravate the phenotype of the long-chain acyl-CoA dehydrogenase KO mouse

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Abstract

Secondary carnitine deficiency is commonly observed in inherited metabolic diseases characterized by the accumulation of acylcarnitines such as mitochondrial fatty acid oxidation (FAO) disorders. It is currently unclear if carnitine deficiency and/or acylcarnitine accumulation play a role in the pathophysiology of FAO disorders. The long-chain acyl-CoA dehydrogenase (LCAD) KO mouse is a model for long-chain FAO disorders and is characterized by decreased levels of tissue and plasma free carnitine. Tissue levels of carnitine are controlled by the SLC22A5, the plasmalemmal carnitine transporter. Here, we have further decreased carnitine availability in the LCAD KO mouse through a genetic intervention by introducing one defective *Slc22a5* allele (*jvs*). *Slc22a5* haploinsufficiency decreased free carnitine levels in liver, kidney and heart of LCAD KO animals. The resulting decrease in the tissue long-chain acylcarnitines levels had a similar magnitude as the decrease in free carnitine. Levels of cardiac deoxycarnitine, a carnitine biosynthesis intermediate, were elevated due to *Slc22a5* haploinsufficiency in LCAD KO mice. A similar increase in heart and muscle deoxycarnitine was observed in an independent

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Conception and design of the work described: SMH
Acquisition of data: PRR, NvV, SMH
Analysis and interpretation of data: PRR, CY, NvV, FMV, SMH
Reporting of the work described: SMH
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Ethics statement

All animal experiments were approved by the IACUC of the Icahn School of Medicine at Mount Sinai (# IACUC-2014–0100) or the institutional review board for animal experiments at the Academic Medical Center, Amsterdam, and comply with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Declaration of interests and competing interests

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experiment using *Slc22a5*$^{jvs/jvs}$ mice. Cardiac hypertrophy, fasting-induced hypoglycemia and increased liver weight, the major phenotypes of the LCAD KO mouse, were not affected by *Slc22a5* haploinsufficiency. This may suggest that secondary carnitine deficiency does not play a major role in the pathophysiology of these phenotypes. Similarly, our data do not support a major role for toxicity of long-chain acylcarnitines in the phenotype of the LCAD KO mouse.

Keywords

carnitine; haploinsufficiency; fatty acid oxidation disorders; modifier; biosynthesis

Introduction

Carnitine is an essential molecule for mitochondrial long-chain fatty acid β-oxidation (FAO) because it enables the translocation of fatty acid intermediates from the cytosol to the mitochondria via the carnitine cycle. Patients with an inborn error of FAO may develop secondary carnitine deficiency. This is likely explained by sequestration of free carnitine as acylcarnitines derived from the accumulating acyl-CoAs followed by tissue efflux of these acylcarnitines and possibly subsequent loss via biliary and urinary excretion. It is currently unclear if carnitine deficiency and/or acylcarnitine accumulation play a role in the pathophysiology of FAO disorders. Nevertheless, many physicians consider carnitine supplementation a mainstay in treatment in particular if free carnitine is very low.

SLC22A5 (also known as OCTN2) is the high affinity carnitine transport responsible for tissue carnitine uptake. Primary carnitine deficiency is a rare FAO disorder caused by biallelic mutations in *SLC22A5*. Carnitine supplementation is essential for the treatment of these patients. Interestingly, heterozygotes carrying one *SLC22A5* mutation have a decreased capacity to accumulate carnitine and increased urinary carnitine loss (Scaglia et al 1998). As a consequence carriers for pathogenic *SLC22A5* mutations have reduced plasma carnitine levels (Scaglia et al 1998). The carrier frequency for pathogenic *SLC22A5* mutations is estimated to vary from 1 in 9 (Faroe Islands) to 1 in 100 and 1 in 188 (Japan and USA, respectively) (Longo 2016). Moreover, genome-wide association studies (GWAS) have demonstrated that common variation at the *SLC22A5* locus also determines circulating levels of carnitine and several acylcarnitines (Shin et al 2014; Burkhardt et al 2015). This metabolite GWAS locus overlaps with expression quantitative trait loci (cis-eQTL) for *SLC22A5*, and is therefore likely mediated via differences in *SLC22A5* expression level (Shin et al 2014). Pathogenic and common *SLC22A5* variants are therefore also likely to affect carnitine levels in patients with a FAO disorder not due to primary carnitine deficiency. Here we address the question if *SLC22A5* haploinsufficiency can affect the disease course in patients with a FAO disorder.

The juvenile visceral steatosis (jvs) mouse is a spontaneous mutant isolated from the C3H.OH strain that has all the characteristics of human primary carnitine deficiency (Koizumi et al 1988). Indeed, jvs mice harbor a loss-of-function mutation in *Slc22a5* (c.1055T>G or p.L352R) (Nezu et al 1999). Similar to humans, mice heterozygous for the *jvs* allele (*Slc22a5*$^{jvs/+}$) have a biochemical phenotype demonstrating a clear gene dose

effect on carnitine transport activity and as a consequence plasma and tissue acylcarnitines (Horiuchi et al 1994; Hashimoto et al 1998; Lahjouji et al 2002; Takahashi et al 2007). Importantly, this illustrates that *Slc22a5*$^{jvs/+}$ mice can be used as a model to mimic variation at the *SLC22A5* locus.

The long-chain acyl-CoA dehydrogenase (LCAD) KO (*Acadl*$^{-/-}$) mouse is considered a model for human long-chain FAO disorders such as very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, because it recapitulates prominent symptoms including fasting-induced hypoketotic hypoglycemia and cardiac hypertrophy (Kurtz et al 1998; Bakermans et al 2011; Houten et al 2013). Here, we have genetically reduced carnitine availability in the LCAD KO mouse by introducing one *jvs* allele, and evaluated changes in the tissue and plasma acylcarnitine profile and the phenotypic presentation. We also studied the effect of biological sex on these parameters.

Material and Methods

Animal experiments

All animal experiments were approved by the IACUC of the Icahn School of Medicine at Mount Sinai (# IACUC-2014–0100) or the institutional review board for animal experiments at the Academic Medical Center, Amsterdam (the Netherlands), and comply with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). We have been backcrossing the *jvs* allele to C57BL/6 in order to obtain a congenic mouse line. The C57BL/6 congenic *Slc22a5*$^{jvs/+}$ were crossed with LCAD KO mice (B6.129S6-*Acadl*^{tm1UAB}/Mmmh (Kurtz et al 1998)) in order to generate *Slc22a5*$^{jvs/+}$ *Acadl*$^{+/-}$ animals on a pure C57BL/6 background (10 to 13 generations of backcrossing). We then crossed these double heterozygous mice with *Acadl*$^{+/-}$ animals in order to generate a progeny containing *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ animals (Fig. S1). The *Acadl* allele was genotyped using a SNP-based genotyping assay (Luther et al 2012) and the *jvs* allele was genotyped and confirmed after necropsy using a PCR-RFLP assay (Nezu et al 1999). The experimental cohort consisted of 20 wild type (WT) animals (of which 12 were males), 12 *Slc22a5*$^{jvs/+}$ *Acadl*$^{+/-}$ (5 males), 19 *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ (8 males) and 17 *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ (8 males) animals, and samples were collected over a period of almost 2 years (August 2016 to July 2018). Mice were on a normal chow diet (PicoLab Rodent Diet 20, LabDiet) and studied at 6 months of age. Blood glucose was measured after overnight food withdrawal using Bayer Contour blood glucose strips. Mice were then euthanized by exposure to CO_2 after which blood was collected for the preparation of EDTA plasma. Organs were snap frozen in liquid nitrogen and stored at −80°C for future analysis.

Until 7 weeks of age, *Slc22a5*$^{jvs/jvs}$ (C3H.OH JVS) were supplemented with L-carnitine in drinking water (300mg/L) or via intraperitoneal injection of 5μmol of L-carnitine HCl dissolved and neutralized in physiological saline (every other day from 5 days of age). At 8 weeks of age, WT and *Slc22a5*$^{jvs/jvs}$ were anesthetized with isoflurane and blood was collected by cardiac puncture. Tissues were collected and snap frozen in liquid nitrogen and stored at −80°C for future analysis.

Immunoblot analysis

Lyophilized mouse hearts were solubilized in RIPA buffer supplemented with protease and phosphatase inhibitors (Pierce) followed by sonication and centrifugation (10 minutes at 12,000 xg at 4°C). Protein concentration was quantified by the BCA method. Proteins were separated on Bolt® 4–12% or 8% Bis-Tris Plus gels (Invitrogen, Thermo Fisher Scientific Inc.), blotted onto a nitrocellulose membrane and detected using the following primary antibodies: BNP (1:500, bs-2207R) from Bioss antibodies (Woburn, MA), LCAD (1:2,000, 17256–1-AP) from Proteintech (Rosemont, IL), α-SMA (1:1,000, 19245) from Cell Signaling Technology (Danvers, MA), and α-tubulin (1:2,000, 32–2500) from Invitrogen, Thermo Fisher Scientific Inc. The MYL1 (1:130, F310) and MYH7 (1:85, A4.480) monoclonal antibodies were developed by F.E. Stockdale and H.M. Blau (Stanford University) and obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. Proteins were visualized with goat anti-mouse and goat anti-rabbit secondary antibodies IRDye 800CW and IRDye 680RD (926–32210, 926–68070, 926–32211, 926–68071) from LI-COR, and Alexa Fluor® 790 AffiniPure Goat Anti-Mouse IgG light chain specific secondary antibody (115-655-174) from Jackson (West Grove, PA). Equal loading was verified by Ponceau S staining and the α-tubulin signal. Band intensity was quantified using Image Studio Lite software version 5.2 (LI-COR).

Metabolite analysis

Plasma acylcarnitines were measured after derivatization to butylesters according to a standardized protocol. Tissue acylcarnitines were measured in freeze-dried tissue samples after derivatization to propylesters essentially as described (van Vlies et al 2005). Samples were analyzed on an Agilent 6460 Triple Quadrupole Mass Spectrometer by the Mount Sinai Biochemical Genetic Testing Lab (Now Sema4). Carnitine biosynthesis intermediates were measured by the Laboratory Genetic Metabolic Diseases (Amsterdam, The Netherlands) essentially as described (Vaz et al 2002).

Metabolomics

Freeze-dried heart (~30mg) samples from 8 females from each of the following experimental groups (WT, *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{tvs/+}$ *Acadl*$^{-/-}$) were shipped to Metabolon (Research Triangle Park, NC) for global metabolite profiling (mView). To select this subset of mice, we picked samples with plasma free carnitine concentrations around the average of the group leaving out the samples with the highest and lowest values. For the analysis, proteins were precipitated with methanol followed by centrifugation. The resulting extract was analyzed by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one RP/UPLC-MS/MS with negative ion mode ESI and by one HILIC/UPLC-MS/MS with negative ion mode ESI essentially as described previously (Evans et al 2014; Miller et al 2015).

Statistics

Using 2way ANOVA (GraphPad Prism 6), we analyzed the effects of genotype (G), sex (S) and their interaction (I) on body, heart and liver weight, blood glucose, and plasma and

tissue acylcarnitines. We used the Sidak's multiple comparisons test to further evaluate differences between *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ animals. The metabolomics data were analyzed as fold-change to the control group with associated p- and q-values in a Welch's Two-Sample t-Test. P values in the figures are indicated as follows: NS not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.

Results

Characterization of Slc22a5$^{jvs/+}$ Acadl$^{-/-}$ mice

Slc22a5$^{jvs/+}$ *Acadl*$^{+/-}$ animals on a pure C57BL/6 background were crossed with *Acadl*$^{+/-}$ animals in order to generate a progeny containing *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ animals as well as control animals. The distribution of genotypes in this progeny was not Mendelian with an underrepresentation of *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ animals (Fig. S1). This is in line with the reported gestational loss in the LCAD KO mouse (Berger and Wood 2004). Consistent with previous reports, blood glucose, heart weight and liver weight were affected by genotype (Kurtz et al 1998; Bakermans et al 2011; Houten et al 2013)(Fig. 1A, Table S1). Whereas heart weight was increased in LCAD KO mice, water content was decreased. No differences were observed between *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ animals. Except for liver weight, none of these phenotypes were affected by sex.

In order to assess if *Slc22a5* haploinsufficiency caused remodeling of the hypertrophied LCAD KO heart, we studied the protein expression of brain natriuretic peptide (BNP; NPPB), myosin light chain 1 (MYL1), myosin heavy chain 7 (MYH7, β-myosin heavy chain) and α-SMA (ACTA2, α-smooth muscle actin)(Fig. 1B). BNP and MYH7 are well-established markers of cardiac remodeling during pathological hypertrophy. α-SMA is a marker to detect the presence of myofibroblasts in cardiac fibrosis. The role of MYL1 in cardiac remodeling is unknown, but we have noted that its expression is decreased in LCAD KO hearts. We did not detect MYH7 protein in any of the WT and LCAD KO heart samples, whereas it was present in a heart sample from a mouse subjected to transverse aortic constriction (TAC; positive control). Expression of BNP was comparable between samples, whereas expression of α-SMA was more variable. MYL1 expression was decreased in LCAD KO samples. Importantly, we found that none of these markers were different between *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ animals. These data confirm that cardiac hypertrophy in the LCAD KO mouse appears non-pathological and is not affected by *Slc22a5* haploinsufficiency.

Slc22a5 haploinsufficiency limits carnitine availability in LCAD KO mice

We determined acylcarnitine profiles in plasma, liver, kidney, heart and soleus muscle of all mice (Table S1). Free carnitine was strongly affected by genotype (Fig. 2). In kidney, heart, soleus and plasma free carnitine mainly decreased as a consequence of the LCAD KO thus reflecting secondary carnitine deficiency. The liver deviated from this pattern, as most of the effect of genotype appears to be caused by the *jvs* allele. Free carnitine was significantly lower in liver, kidney and heart of *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ animals when compared to *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ animals. Effects of sex on free carnitine were noted in kidney and

soleus. In soleus the interaction term was significant indicating that genotype does not have the same effect in males versus females.

Acetylcarnitine is a major reservoir of carnitine. This metabolite was decreased in LCAD KO animals with pronounced reductions in kidney, heart and plasma (Fig. S2). Acetylcarnitine was not significantly affected by the *jvs* allele. Effects of sex were present in most analyzed compartments, but for kidney acetylcarnitine, genotype and sex had a significant interaction term.

Slc22a5 haploinsufficiency limits long-chain acylcarnitine accumulation in LCAD KO mice

C14:1-carnitine is the primary accumulating acylcarnitine in plasma and tissues of LCAD KO mice. Consistently, genotype explains most variability in plasma and all other tissues analyzed (Fig. 3). The *jvs* allele significantly decreased C14:1-carnitine accumulation in plasma and all analyzed tissues of *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ animals when compared to *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ animals. This indicates that decreased carnitine availability can lower the acylcarnitine accumulation.

We next focused on C16- and C18:1-carnitine, which are derived from dietary and stored fatty acids. Again genotype had the most pronounced effect on C16- and C18:1-carnitine, but the direction of change depended on the tissue (Fig. 3, S3). Whereas these long-chain acylcarnitines increased in plasma and liver of LCAD KO mice, they decreased in kidney, heart and soleus muscle. The *jvs* allele significantly decreased the levels of C16-carnitine in liver and kidney of the *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ animals. For C18:1-carnitine, this decrease was also significant in plasma. Changes in total long-chain acylcarnitines (sum of all C14, C16 and C18) were comparable to those of C16- and C18:1-carnitine (Fig. S3). For all long-chain acylcarnitines, effects of sex were noted in liver and kidney with a significant interaction term in liver only.

Slc22a5 haploinsufficiency affects carnitine biosynthesis intermediates

In order to evaluate the impact of *Slc22a5* haploinsufficiency in a non-biased way, we performed metabolite profiling in heart from WT, *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ animals. Within the mouse heart, 726 known metabolites were detected and quantified. Many biochemicals were significantly altered in LCAD KO hearts when compared to hearts from WT animals. These include previously reported metabolites such as carbohydrates, free fatty acids, acylcarnitines, TCA cycle intermediates and amino acids (Bakermans et al 2013; Houten et al 2013). There are also many metabolites that were previously not reported to be affected in the LCAD KO mouse model including 7-ketodeoxycholate, several acylcholines and glutathione adducts, pipecolate and hypotaurine. A complete overview of the metabolomics data is provided in Table S2.

A direct comparison of *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ hearts yielded no metabolites with a q-value < 0.05, but 36 metabolites were nominally significant ($P < 0.05$). Deoxycarnitine (gamma-butyrobetaine), the direct precursor of carnitine, was the most significantly changed metabolite and increased 1.4-fold in *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ hearts (Fig. 4A, $P = 0.0017$). The second most significantly changed metabolite was N,N,N-trimethyl-5-aminovalerate, which decreased 1.9-fold (Fig. 4A, $P = 0.0035$). Intriguingly, this metabolite

is only one methylene group longer than deoxycarnitine. Trimethyllysine, also a carnitine biosynthesis intermediate, did not differ between groups. Carnitine itself is the 11th most significantly changed metabolite with a 1.2-fold change. The clustering of carnitine and 2 related metabolites at the top of this p-value-ranked list ranked illustrates that the changes in these metabolites are likely real. To further illustrate this, we calculated the carnitine/deoxycarnitine ratio, which was 1.6-fold decreased in *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$. The Benjamini-Hochberg Adjusted P value for this ratio was significant (q = 0.009).

Because of the changes in cardiac deoxycarnitine, we next quantified carnitine biosynthesis intermediates in plasma. The concentration of trimethyllysine did not differ between groups (Table S1). The deoxycarnitine concentration was 2.7-fold decreased in LCAD KO mice, but there was no difference between *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ mice (Fig. 4B).

To investigate whether the increased levels of cardiac deoxycarnitine observed in *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ mice was dependent on the *Acadl*$^{-/-}$ genotype, we also measured carnitine biosynthesis intermediates in tissues and plasma of a separate cohort of WT and *Slc22a5*$^{jvs/jvs}$ mice. Small changes in tissue levels of trimethyllysine were noted with a decrease in liver and an increase in muscle of *Slc22a5*$^{jvs/jvs}$ mice. Levels of trimethyllysine were not changed in kidney, heart and plasma (Figure S4). Deoxycarnitine levels were decreased in liver, kidney and plasma and increased in heart and muscle of *Slc22a5*$^{jvs/jvs}$ mice (Fig. 4C). This indicates that cardiac and possibly muscle deoxycarnitine levels increase with diminished SLC22A5 function.

Discussion

Secondary carnitine deficiency due to acylcarnitine accumulation is commonly observed in inborn errors of acyl-CoA metabolism such as FAO disorders. Accordingly, LCAD KO mice, which are utilized as a model for long-chain FAO disorders, have decreased levels of tissue and plasma free carnitine. Here, we have further decreased carnitine availability in the LCAD KO mouse through a genetic intervention by introducing one mutant *Slc22a5* allele (*jvs*). *Slc22a5* haploinsufficiency decreased free carnitine in liver, kidney and heart of LCAD KO animals. The resulting decrease in the tissue long-chain acylcarnitines levels was overall proportional. Cardiac hypertrophy, fasting-induced hypoglycemia and increased liver weight, the major phenotypes of the LCAD KO mouse, were not affected by *Slc22a5* haploinsufficiency. This may suggest that secondary carnitine deficiency does not play a major role in the pathophysiology of these phenotypes. Similarly, our data do not support a major role for toxicity of long-chain acylcarnitines in the phenotype of the LCAD KO mouse.

An untargeted metabolomics analysis of heart samples revealed only subtle changes between *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ animals, which further illustrates the limited impact of *Slc22a5* haploinsufficiency on the biochemical phenotype of the LCAD KO mouse. The most pronounced changes were observed in the levels of cardiac carnitine and its biosynthetic precursor deoxycarnitine. We noted an increase in the levels of deoxycarnitine and a decrease in the carnitine/deoxycarnitine ratio in *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$

hearts when compared to *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ hearts. We observed a similar phenomenon in mice with a complete defect in SLC22A5 (*Slc22a5*$^{jvs/jvs}$). Deoxycarnitine was increased in heart and muscle of this mouse model for primary carnitine deficiency.

Gamma-butyrobetaine hydroxylase (BBOX1) catalyzes the conversion of deoxycarnitine into carnitine, the ultimate step in carnitine biosynthesis. It is well established that in humans BBOX1 is exclusively expressed in kidney, liver and brain (Vaz et al 1998). In rodents, BBOX1 expression is even more restricted and is largely liver-specific (van Vlies et al 2006). Given the absence of BBOX1 in the heart and absence of changes in cardiac trimethyllysine levels, the increase in deoxycarnitine in *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ hearts is most likely due to impaired efflux. Intriguingly, N,N,N-trimethyl-5-aminovalerate, a molecule structurally similar to deoxycarnitine, was decreased in *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ hearts. Not much is known about N,N,N-trimethyl-5-aminovalerate except that is a biomarker for milk intake and a predictor for the development of microalbuminuria (Pallister et al 2017; Haukka et al 2018). Therefore the main difference between N,N,N-trimethyl-5-aminovalerate and deoxycarnitine is their origin i.e. exogenous versus endogenous, respectively. Given their structural similarity, we speculate that these two metabolites utilize the same membrane transport mechanism. Both SLC6A13 and SLC22A5 haven been demonstrated to transport deoxycarnitine (Tamai et al 1998; Fujita et al 2009; Nakanishi et al 2011). Of these transporters, only SLC22A5 is expressed in the heart. Interestingly, SLC22A5 has been demonstrated to function as an antiporter exchanging carnitine and related molecules such as deoxycarnitine stimulated by external Na^+ (Ohashi et al 2001; Pochini et al 2004). We speculate that SLC22A5 can exchange N,N,N-trimethyl-5-aminovalerate for deoxycarnitine. A defect in this exchange mechanism due to *Slc22a5* haploinsufficiency could explain the increased heart deoxycarnitine and decreased heart N,N,N-trimethyl-5-aminovalerate. Based on this observation, we suggest that SLC22A5 plays a role in deoxycarnitine efflux from the heart and possibly other tissues such as skeletal muscle. Future in vitro studies should address this hypothesis.

Strengths of this study include the biochemical analyses in a relatively large cohort of male and female mice, which enabled us to analyze the effects of genotype, sex and their interaction. Sexual dimorphism in plasma acylcarnitine concentrations have been reported (Siegert et al 2012; Tucci et al 2015; Muilwijk et al 2018), but effects of sex on tissue (acyl)carnitine levels are unexplored. We observed sexual dimorphism for the levels of many acylcarnitines in particular in the kidney, an organ known to have high degree of sexually dimorphic gene expression (Si et al 2009). Among the limitations is the relatively mild phenotype of the LCAD KO mouse model, which includes fasting-induced hypoketotic hypoglycemia, cardiac hypertrophy and increased liver weight. In contrast to the human long-chain FAO disorders, these phenotypes generally do not progress to a life-threatening condition. We show that *Slc22a5* haploinsufficiency did not affect fasting-induced hypoglycemia and increased liver weight. Cardiac mass tended to be lower in *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ when compared to *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ mice, but this difference did not reach statistical significance. Moreover, cardiac weight in *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ mice was still increased when compared to WT mice. Therefore, we conclude that carnitine deficiency does not play a major role in the pathophysiology of these LCAD KO phenotypes. Similarly, our data do not support a major role for toxicity of long-chain acylcarnitines. The LCAD KO

mouse does not present with skeletal myopathy and/or rhabdomyolysis. Therefore our study does not address the skeletal muscle associated pathology. It is currently unclear how relevant our findings are to patients and patient management. A second limitation is that we did not study cardiac function or ECG and cannot exclude that *Slc22a5* haploinsufficiency affects these parameters. Immunoblotting for markers of cardiac remodeling indicated the non-pathological nature of the hypertrophy in the LCAD KO mouse, which was not affected by *Slc22a5* haploinsufficiency.

In conclusion, *Slc22a5* haploinsufficiency leads to small and proportional changes in the plasma and tissue acylcarnitine profile of an animal model for long-chain FAO disorders and does not appear to further derail metabolic processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Take home message

Slc22a5 haploinsufficiency does not aggravate the clinical phenotype of a mouse model for mitochondrial fatty acid oxidation disorders

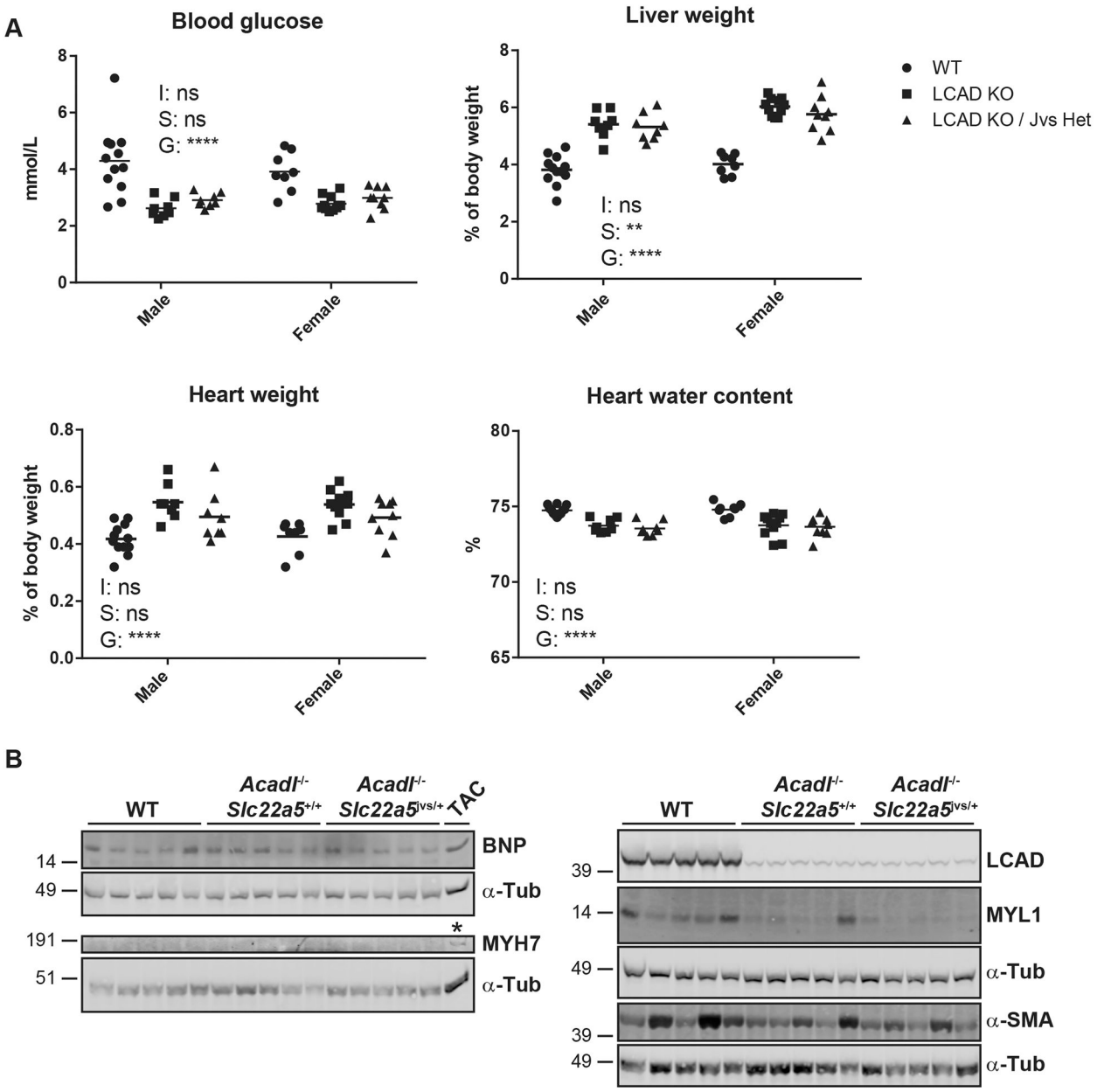

Figure 1.

Characterization of LCAD KO phenotypes in *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ mice. (A) Individual values and the average for blood glucose, liver weight, heart weight and heart water content are graphed for males and females separately. The result of the 2way ANOVA is displayed within the graph: I denotes the significance of the interaction term, S denotes the significance of the effect of sex and G denotes the significance of the effect of genotype. The results of Sidak's multiple comparisons test is only displayed if the difference between *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ animals is significant. (B) Immunoblots of markers for cardiac remodeling in WT, *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ animals (5 male mice per group). Each lane contains 50 μg of total heart protein. The last lane of the BNP and MYH7 immunoblots includes a sample from a TAC mouse heart as

positive control. The MYH7 band in the TAC sample was observed in 3 independent immunoblots, whereas all other samples were repeatedly negative. For each separate blot the α-Tubulin loading control is displayed. The position of the molecular weight markers is indicated in kDa. Quantification of relative protein levels did not reveal any differences between the *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{ivs/+}$ *Acadl*$^{-/-}$ animals.

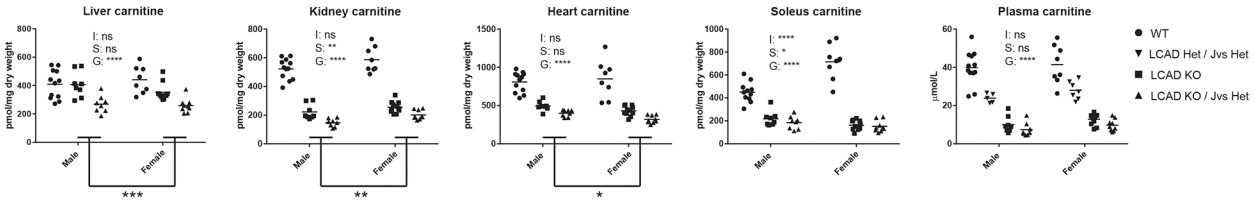

Figure 2.

Free carnitine levels. Free carnitine levels in liver, kidney, heart, soleus muscle and plasma of WT, *Slc22a5*$^{\text{jvs/+}}$ *Acadl*$^{+/-}$ (plasma only), *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{\text{jvs/+}}$ *Acadl*$^{-/-}$ mice. Individual values and the average for each parameter are graphed for males and females separately. The result of the 2way ANOVA is displayed within the graph: I denotes the significance of the interaction term, S denotes the significance of the effect of sex and G denotes the significance of the effect of genotype. The results of Sidak's multiple comparisons test is only displayed if the difference between *Slc22a5*$^{\text{jvs/+}}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ animals is significant.

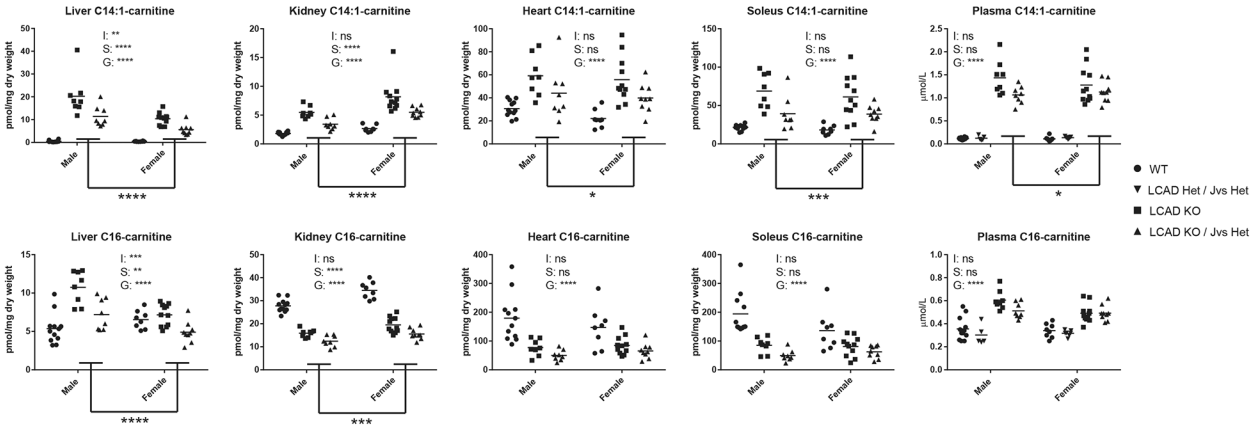

Figure 3.

Long-chain acylcarnitine levels. C14:1- and C16-carnitine levels in liver, kidney, heart, soleus muscle and plasma of WT, *Slc22a5*$^{\text{jvs/+}}$ *Acadl*$^{+/-}$ (plasma only), *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{\text{jvs/+}}$ *Acadl*$^{-/-}$ mice. Individual values and the average for each parameter are graphed for males and females separately. The result of the 2way ANOVA is displayed within the graph: I denotes the significance of the interaction term, S denotes the significance of the effect of sex and G denotes the significance of the effect of genotype. The results of Sidak's multiple comparisons test is only displayed if the difference between *Slc22a5*$^{\text{jvs/+}}$ *Acadl*$^{-/-}$ and *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ animals is significant.

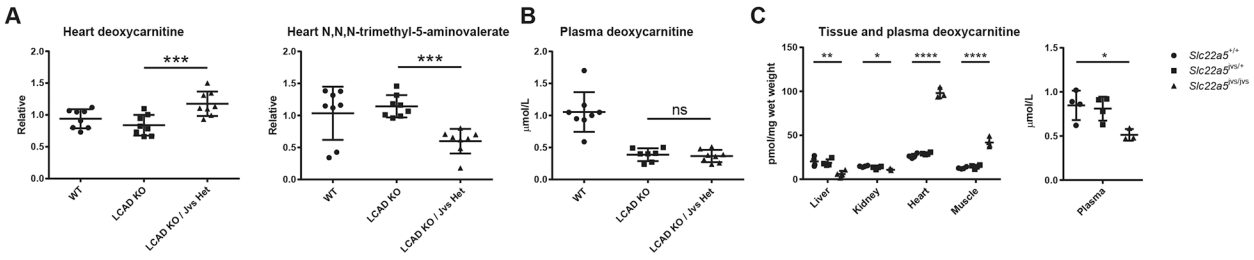

Figure 4.

Carnitine biosynthesis intermediates. A. Heart deoxycarnitine (8 females per group) and N,N,N-trimethyl-5-aminovalerate (8 females per group). The heart data are relative with the median equal to 1. B. Plasma deoxycarnitine in WT (n=8, 4 males), *Slc22a5*$^{+/+}$ *Acadl*$^{-/-}$ (n=7, 4 males) and *Slc22a5*$^{jvs/+}$ *Acadl*$^{-/-}$ mice (n=8, 4 males). C. Tissue levels and plasma concentration of deoxycarnitine in *Slc22a5*$^{+/+}$ (4 animals), *Slc22a5*$^{jvs/+}$ (4 animals) and *Slc22a5*$^{jvs/jvs}$ mice (3 or 4 animals). The result of the 1way ANOVA is displayed within the graph.