Effect of lifelong carnitine supplementation on plasma and tissue carnitine status, hepatic lipid metabolism and stress signalling pathways and skeletal muscle transcriptome in mice at advanced age

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Abstract

While strong evidence from clinical studies suggests beneficial effects of carnitine supplementation on metabolic health, serious safety concerns associated with carnitine supplementation have been raised from studies in mice. Considering that the carnitine doses in these mice studies were up to 100 times higher than those used in clinical studies, the present study aimed to address possible safety concerns associated with long-term supplementation of a carnitine dose used in clinical trials. Two groups of NMRI mice were fed either a control or a carnitine-supplemented diet (1 g/kg diet) from weaning to 19 months of age, and parameters of hepatic lipid metabolism and stress signalling and skeletal muscle gene expression were analysed in the mice at 19 months of age. Concentrations of free carnitine and acetylcarnitine in plasma and tissues were higher in the carnitine than in the control group ($P<0.05$). Plasma concentrations of free carnitine and acetylcarnitine were higher in mice at adult age (10 and 15 months) than at advanced age (19 months) ($P<0.05$). Hepatic mRNA and protein levels of genes involved in lipid metabolism and stress signalling and hepatic and plasma lipid concentrations did not differ between the carnitine and the control group. Skeletal muscle transcriptome analysis in 19-month-old mice revealed only a moderate regulation between carnitine and control group. Lifelong carnitine supplementation prevents an age-dependent impairment of plasma carnitine status, but safety concerns associated with long-term supplementation of carnitine at doses used in clinical trials can be considered as unfounded.

Key words: Carnitine supplementation: Aged mice: Liver: Skeletal muscle

L-Carnitine has long been known to be vital to all cells containing mitochondria due to its ability to shuttle long-chain activated fatty acids (acyl-CoA) as acylcarnitines from the cytosol into the mitochondrial matrix, where fatty acid β-oxidation takes place[1]. However, carnitine is also involved in carbohydrate oxidation because it is also required for the efflux of acyl groups as acylcarnitines out of mitochondria, thereby reducing the intramitochondrial acetyl-CoA:CoA ratio[2]. As a consequence, acetyl-CoA-mediated inhibition of the activity of pyruvate dehydrogenase, a key metabolic enzyme of glucose utilisation, is relieved and glucose oxidation is stimulated[3,4]. This explains, at least partially, that a reduced carnitine status in diabetic subjects[5–7] is associated not only with impaired fatty acid oxidation but also with perturbations of glucose utilisation and insulin sensitivity. In line with this, a large number of studies in humans and animals revealed that supplementation of carnitine or

carnitine derivatives (e.g. acetylcarnitine and propionylcarnitine) improves the carnitine status and causes an improvement of whole-body glucose tolerance and insulin sensitivity of insulin-dependent tissues such as skeletal muscle and heart, in particular during insulin-resistant states[8]. In addition, clinical studies even demonstrated that myocardial carnitine deficiency is associated with cardiomyopathy[9,10], and carnitine supplementation restores tissue levels of carnitine and improves heart function[9,11]. Moreover, a significant number of clinical studies showed an improvement of quality of life measures, nutritional status and body condition and a reduction of fatigue-related symptoms in patients with chronic diseases such as cancer, chronic kidney disease, HIV, or hepatic encephalopathy due to carnitine supplementation[12–16]. Such patients frequently exhibit reduced plasma carnitine levels[17–19] as a consequence of different reasons, such as a reduced food intake, impaired

intestinal carnitine absorption as a side effect of pharmaco-therapy and chemotherapy and loss of carnitine during hemodialysis as in the case of chronic kidney disease patients.

Despite this large body of evidence from clinical studies[8–16] that an improvement of carnitine status has beneficial effects on metabolic health, serious safety concerns with carnitine supplementation have been raised based on the recent observation that long-term carnitine supplementation (from weaning to 5 months of age) in a transgenic mouse model of atherosclerosis (*Apoe*$^{-/-}$ mice) promotes atherosclerosis through gut microbiota-dependent formation of trimethylamine and subsequent hepatic conversion into proatherogenic trimethylamine-*N*-oxide[20]. In addition, it has been found that carnitine supplementation to normal mice for 3 months induces oxidative stress, liver inflammation, liver lipid accumulation and even liver damage as well as hypercholesterinaemia[21]. Regarding these reports, the widespread use of carnitine supplements, which are easily distributed over the counter, in both the healthy and ill population might be seen critically. However, it has to be mentioned that the carnitine dose administered to the mice in the aforementioned studies was extremely high. In that study[21], about 200 mg carnitine was taken up daily per mouse from carnitine-supplemented drinking water which related to approximately 4000 mg/kg body weight (BW). The carnitine dose in the study with the *Apoe*$^{-/-}$ mice[20] was even greater than 4000 mg/kg BW considering a carnitine concentration of 1·3 % in the drinking water and an estimated daily water intake of 10 ml by a 5-month-old mouse consuming 3–4 g feed per d. By contrast, in clinical trials, the daily amount of carnitine provided to human subjects is typical between 2 and 6 g[22,23], which relates to a carnitine dose of 30–90 mg/kg BW for a person weighing 65 kg. It might be argued therefore that the detrimental outcomes of carnitine supplementation in the aforementioned mice studies are due to the extremely high dose of carnitine administered.

Thus, in order to address whether safety concerns are founded under conditions of long-term supplementation of a carnitine dose used in clinical trials, the present study aimed to investigate the effect of almost lifelong daily administration of a carnitine-supplemented diet (1 g/kg diet) to mice from weaning to 19 months of age. Considering a BW of 50 g for an adult mice and a daily feed intake of 5 g, the daily intake of this diet results in a daily carnitine dose of 100 mg/kg BW which better reflects the dose used in clinical trials. In order to evaluate possible detrimental effects of carnitine supplementation, we investigated parameters of lipid metabolism and stress signalling pathways (endoplasmic reticulum (ER) stress-induced unfolded protein response, NF-κB, nuclear factor-E2 related factor 2) in the liver and performed a genome-wide transcriptome analysis of skeletal muscle in the mice at advanced age.

Methods

Animals and diets

The animal experiment was approved by the local Animal Care and Use Committee (Regierungspräsidium Giessen; permission no. V 54 – 19 c 20 15 h 02 GI 19/3 kTV 12/2018). All experimental procedures described followed established guidelines for the

Table 1. Composition of the basal natural ingredient diet

Component	g/kg diet
Wheat	429·5
Maize	252
Soyabean extraction meal (44 % crude protein)	200
Wheat bran	40
Soyabean oil	28
Oats	20
Calcium hydrogen phosphate dihydrate	11
Mineral–vitamin mix*	6
Calcium carbonate	6
DL-Methionine	4·5
L-Lysine-HCl	1·5
Choline chloride	1·5

* The mineral–vitamin mix provided the following per kg of diet: Ca, 1·3 g; P, 0·2 g; Na, 1·57 g; Mg, 0·1 g; Fe, 18 mg; Zn, 62 mg; Mn, 18 mg; Cu, 3·6 mg; iodate, 0·39 mg; Se, 0·34 mg; all-*trans*-retinol, 0·72 mg; cholecalciferol, 27·4 μg; menadione sodium bisulphate, 1·07 mg, all-*rac*-α tocopheryl acetate, 18 mg; thiamine-HCl, 3 mg; riboflavin, 2·7 mg; pyridoxine-HCl, 4·9 mg; folic acid, 1·2 mg; nicotinic acid, 11 mg; pantothenic acid, 8 mg; biotin, 0·22 mg; cyanocobalamine, 0·056 mg; choline, 60 mg.

care and handling of laboratory animals[24]. The animal experiment included a total of sixty male, 3-week-old NMRI mice (Harlan Winkelmann). The NMRI mouse is a general-purpose model used in different fields, including toxicology, teratology, pharmacology and physiology, and therefore suitable to study the effects of long-term carnitine supplementation. Mice were housed in groups of five each under controlled conditions (Makrolon cages, lighting from 07.00 to 19.00 hours, 22 ± 1°C ambient temperature, 50–60 % relative humidity). The mice were randomly assigned to two groups (control and carnitine) of thirty mice per group with similar BW in the control group (12·0 (SD 0·2) g) and the carnitine group (12·0 (SD 0·3) g). Mice of the two groups were fed the same basal natural ingredient diet containing all nutrients in accordance with National Research Council recommendations of laboratory rodents[25]. The composition of the basal natural ingredient diet is presented in Table 1. Soyabean extraction meal was used instead of casein due to its low native carnitine concentration. In the control group, the basal natural ingredient diet, which had a low carnitine concentration of <5 mg/kg diet as determined by tandem MS, was fed unsupplemented. In the carnitine group, the basal natural ingredient diet was supplemented with 1000 mg L-carnitine (Carnifeed; Lonza) per kg of diet. The mice had free access to the diets, and food intake was recorded weekly. Water was available *ad libitum* from nipple drinkers during the whole experiment. All animals were assessed visually each day for normal appearance, behaviour and the absence of any signs of impaired health. No adverse events in any of the mice were observed during the whole experiment.

Sample collection

At an age of 10, 15 and 19 months, ten mice of each group were decapitated under CO_2 anaesthesia. Blood was collected into EDTA polyethylene tubes (Sarstedt), and plasma was separated from blood by centrifugation at 1100 **g** at 4°C for 10 min). The liver and an aliquot of skeletal muscle (*Musculus gastrocnemius*) were excised and immediately snap-frozen in liquid N_2. Afterwards, all samples were stored at –80°C pending analysis.

Analysis of carnitine and its precursors in plasma and tissues

Concentrations of free carnitine, acetylcarnitine, γ-butyrobetaine (γ-BB) and trimethyllysine in plasma, liver and *M. gastrocnemius* were determined using tandem MS according to Hirche *et al.*[26].

Analysis of TAG and cholesterol in plasma and liver

Lipids from liver samples were extracted with a mixture of *n*-hexane and isopropanol (3:2, v/v) according to Hara & Radin[27]. Lipid extracts were dried and lipids dissolved with chloroform and Triton X-100 (1:1, v/v) as described by Eder & Kirchgessner[28]. TAG and cholesterol contents were measured using enzymatic reagent kits (Fluitest CHOL, catalogue no. 4241; Fluitest TG, catalogue no. 5741; both from Analyticon Biotechnologies).

Total RNA isolation from liver and skeletal muscle

Total RNA from 10 mg frozen liver aliquots was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA from 30 mg frozen skeletal muscle aliquots was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Afterwards, total RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively, using an Infinite 200M microplate reader equipped with a NanoQuant plate (both from Tecan). The average optical density A260:A280 ratio of all liver total RNA samples was 1·94 (SD 0·01, *n* 10) and 1·92 (SD 0·05, *n* 10) for the control group and the carnitine group, respectively. The average optical density A260:A280 ratio of all skeletal muscle total RNA samples was 1·87 (SD 0·02, *n* 10) and 1·87 (SD 0·02, *n* 10) for the control group and the carnitine group, respectively.

Quantitative real-time PCR analysis

Synthesis of cDNA and qPCR analysis on a Rotor-Gene Q system (Qiagen) were carried out as described recently in detail[29]. Briefly, for qPCR the KAPA SYBR FAST qPCR Mastermix from Peqlab and gene-specific primer pairs from Eurofins MWG Operon were used. Characteristics of primers used for qPCR analysis of hepatic gene expression and for qPCR validation of microarray data in skeletal muscle are shown in online Supplementary Tables S1 and S2, respectively. The qPCR protocol comprised an initial heating step at 95°C for 3 min followed by 35–40 cycles of a three-step reaction (95°C for 3 s, 60°C for 20 s, 72°C for 1 s). The reaction was followed by a melting curve from 50 to 95°C in 5 s increments of 1°C to ensure amplification specificity. All PCR products were separated electrophoretically using a 1·5 % agarose gel stained with GelRed™ nucleic acid gel stain (Biotium) to confirm the amplification of a single PCR product of the expected size. For calculation of mRNA levels Ct-values of reference and target genes were obtained using Rotor-Gene Q Software (Qiagen). Raw Ct-values of reference genes were transformed into relative expression values using the $2^{-\Delta Ct}$ equation for the calculation of normalisation factors. The highest relative value of each gene was set to (1). From these values, the normalisation factor was calculated as the geometric mean of expression data of the three most stable (liver: *Actb*, *Atp5b*, *Rpl13a*; skeletal muscle: *Eif4a2*, *Actb*, *Rpl13a*) out of six potential reference genes according to Vandesompele *et al.*[30]. The stability *M*-values of the three most stable reference genes in liver and skeletal muscle were <0·55 and <0·70, respectively. Ct-values of target genes were also transformed into relative expression values using the $2^{-\Delta Ct}$ equation and were normalised with the individual normalisation factor resulting in relative gene quantities that were used for the statistical analysis.

Immunoblotting

Immunoblotting was carried out as described recently in detail[31]. In brief, homogenates were prepared from frozen liver aliquots using radioimmunoprecipitation assay buffer. From each homogenate, the same amount of protein (30 μg) was separated on 12·5 % SDS-PAGE and electrotransferred to a nitrocellulose membrane. After incubating the membranes overnight at 4°C in blocking solution, membranes were incubated with primary antibodies against ALDH9A1 (polyclonal; Abnova), BBOX1 (monoclonal; Abcam), OCTN2 (polyclonal; LifeSpan Biosciences), glutathione peroxidase (polyclonal; Abcam), heme oxygenase (monoclonal; Abcam) and β-actin (monoclonal; Abcam) as a reference protein to control for adequate normalisation at room temperature. Following a washing step, membranes were incubated with a horseradish-peroxidase-conjugated secondary monoclonal anti-mouse-IgG (Sigma-Aldrich) or polyclonal anti-mouse-IgG antibodies (DakoCytomation) at room temperature and subsequently developed using ECL Plus (GE Healthcare). The signal intensities of specific bands were detected with a Bio-Imaging system (Syngene) and quantified using Syngene GeneTools software (non-linear dynamics). For calculation of protein levels, the band intensity of the proteins of interest was normalised by that of β-actin.

Microarray analysis and bioinformatic analysis of microarray data

For microarray analysis of skeletal muscle, five skeletal muscle total RNA samples were randomly selected each from the control group and the carnitine group. Prior to RNA sample processing, RNA quality was checked using an Agilent 2100 Bioanalyser (Agilent technologies). RNA integrity number values were 7·4 (SD 0·7, *n* 5) and 7·5 (SD 0·3, *n* 5) for the control group and the carnitine group, respectively. RNA samples were processed at the 'Kompetenzzentrum Fluoreszente Bioanalytik' (Regensburg) using Affymetrix GeneChip Mouse Gene 2.0 Sense Target arrays as described[32]. The microarray data have been submitted in MIAME compliant format to the NCBI's Gene Expression Omnibus public repository[33] (GEO accession no. GSE120120). Microarray analysis was carried out using the Robust Multichip Analysis algorithm[34]. Transcripts were defined as differentially expressed when the fold change (FC) between carnitine and control group was >1·2 or <−1·2, and *P* value of the unpaired Student's *t* test was <0·05. In order to extract biological meaning from the list of differentially expressed genes, gene set enrichment analysis

Table 2. Body weight (BW) development and food intake of mice fed a control diet or a diet supplemented with 1000 mg/kg diet ʟ-carnitine from 3 weeks to 19 months of age (*n* 10 mice/group)
(Mean values and standard deviations)

	Control		Carnitine		
	Mean	SD	Mean	SD	*P*
BW at 3 weeks (g)	12·0	0·2	12·0	0·3	0·93
BW at 10 months (g)	56·4	5·4	59·8	4·9	0·16
BW at 15 months (g)	56·2	7·5	53·5	7·9	0·45
BW at 19 months (g)	57·7	4·4	56·4	4·3	0·18
Total BW gain (g)	45·7	4·4	44·5	4·4	0·52
DFI from 3 weeks to 10 months (g)	6·4	0·7	6·3	0·5	0·98
DFI from 10 to 15 months (g)	6·0	0·7	6·2	0·4	0·69
DFI from 15 to 19 months (g)	5·5	0·6	5·8	0·6	0·58

DFI, daily food intake.

(GSEA) was performed separately for the up- and down-regulated genes using Gene Ontology (GO) biological process and GO molecular function terms using the Database for Annotation, Visualisation and Integrated Discovery 6.8 bioinformatic resource[35,36].

Statistical analysis

Data are shown as means and standard deviations. The data were subjected to ANOVA using the Minitab Statistical Software Rel. 13.1 (Minitab). Data considering different ages of the mice (carnitine metabolites and lipids in plasma and tissues) were subjected to two-factorial ANOVA with classification factors being carnitine (Carn), age (Age) and the interaction of both factors (Carn × Age). For statistically significant *F* values, individual means of the treatment groups were compared by Fisher's multiple range test. Corresponding non-parametric tests were used to analyse data with non-normal distributions. Data considering only one age of the mice (transcript measurements, immunoblotting) were analysed by Student's *t* test. Means were considered significantly different for *P*<0·05.

The number of mice per group for detecting a significant difference among groups (main parameter: plasma-free carnitine) was calculated by G*Power (rel. 3.1.2) assuming a hypothesised effect size of 1·35, a type I error of 0·05 (two sided) and a statistical power of 0·80.

Results

Daily food intake, body weight of the mice at 10, 15 and 19 months of age and total body weight gain

Daily food intake during the experiment, BW at 10, 15 and 19 months of age and total BW gain from 3 weeks to 19 months of age did not differ between the control and the carnitine group (Table 2).

Concentrations of carnitine and its precursors in plasma and tissues of the mice at 10, 15 and 19 months of age

The concentrations of free carnitine, acetylcarnitine and the sum of free carnitine and acetylcarnitine in plasma, liver and skeletal muscle were higher in mice of the carnitine group than in those of the control group (*P*<0·05, Fig. 1(A)–(C)). Plasma concentration of free carnitine and the sum of free carnitine and acetylcarnitine were higher in mice at adult age (10 and 15 months) than in mice at advanced age (19 months) (*P*<0·05). While the concentrations of free carnitine and the sum of free carnitine and acetylcarnitine in liver and skeletal muscle were not influenced by the age of the mice, the concentration of acetylcarnitine in liver and muscle was influenced by the age of the mice. Muscle acetylcarnitine concentration was decreased in mice at advanced age (19 months) compared with mice at adult age (10 and 15 months) (*P*<0·05). Hepatic acetylcarnitine concentration, which made up less than 2 % of the sum of free carnitine and acetylcarnitine, was increased in mice at advanced age (19 months) compared with mice at adult age (10 and 15 months) (*P*<0·05).

The concentrations of γ-BB in plasma, liver and skeletal muscle were 4·5-, 4·5- and 2·3-fold higher in mice of the carnitine group than in those of the control group (*P*<0·05, Fig. 2(A)–(C)). The age of the mice had no influence on the concentrations of γ-BB in all tissues investigated. Plasma concentration of trimethyllysine was 15–20 % higher in mice of the carnitine group than in those of the control group (*P*<0·05, Fig. 2(A)) but was not influenced by the age of the mice.

Concentrations of TAG and cholesterol in plasma and liver of the mice at 10 and 19 months of age

The concentrations of TAG and cholesterol in plasma and liver did not differ between mice of the carnitine group and those of the control group at 10 and 19 months of age (online Supplementary Fig. S1).

Relative mRNA and protein levels of key genes involved in lipid metabolism in the liver of mice at 19 months of age

Relative mRNA concentrations of PPARα-regulated genes involved in lipid catabolism in the liver of mice at an age of 19 months either did not differ between the two groups (acyl-CoA dehydrogenase medium chain (*Acadm*), solute carrier family 25 member 20/carnitine/acylcarnitine translocase (*Slc25a20/Cact*), solute carrier family 27 member 1/fatty acid transporter 1 (*Slc27a1/Fatp1*) and solute carrier family 22 member 5/organic cation transporter 2 (*Slc22a5/Octn2*)) or were significantly higher (aldehyde dehydrogenase 9 family member A1 (*Aldh9a1*)) or tended to be higher (γ-butyrobetaine hydroxylase 1 (*Bbox1*)) in the carnitine group than in the control group (online Supplementary Table S3). The relative mRNA concentrations of key genes involved in lipid synthesis (acetyl-CoA carboxylase α (*Acaca*), fatty acid synthase (*Fasn*), glycerol-3-phosphate acyltransferase, mitochondrial (*Gpam*) and stearoyl-CoA desaturase (*Scd1*)), which are under the control of sterol regulatory element-binding proteins (SREBP), in the liver of mice at an age of 19 months did not differ between the carnitine group and the control group (online Supplementary Table S3). Relative protein concentrations of ALDH9A1, BBOX1 and OCTN2, which are involved in the regulation of carnitine homeostasis, in the liver of mice at an age of 19 months were not different between the carnitine group and the control group (online Supplementary Fig. S2).
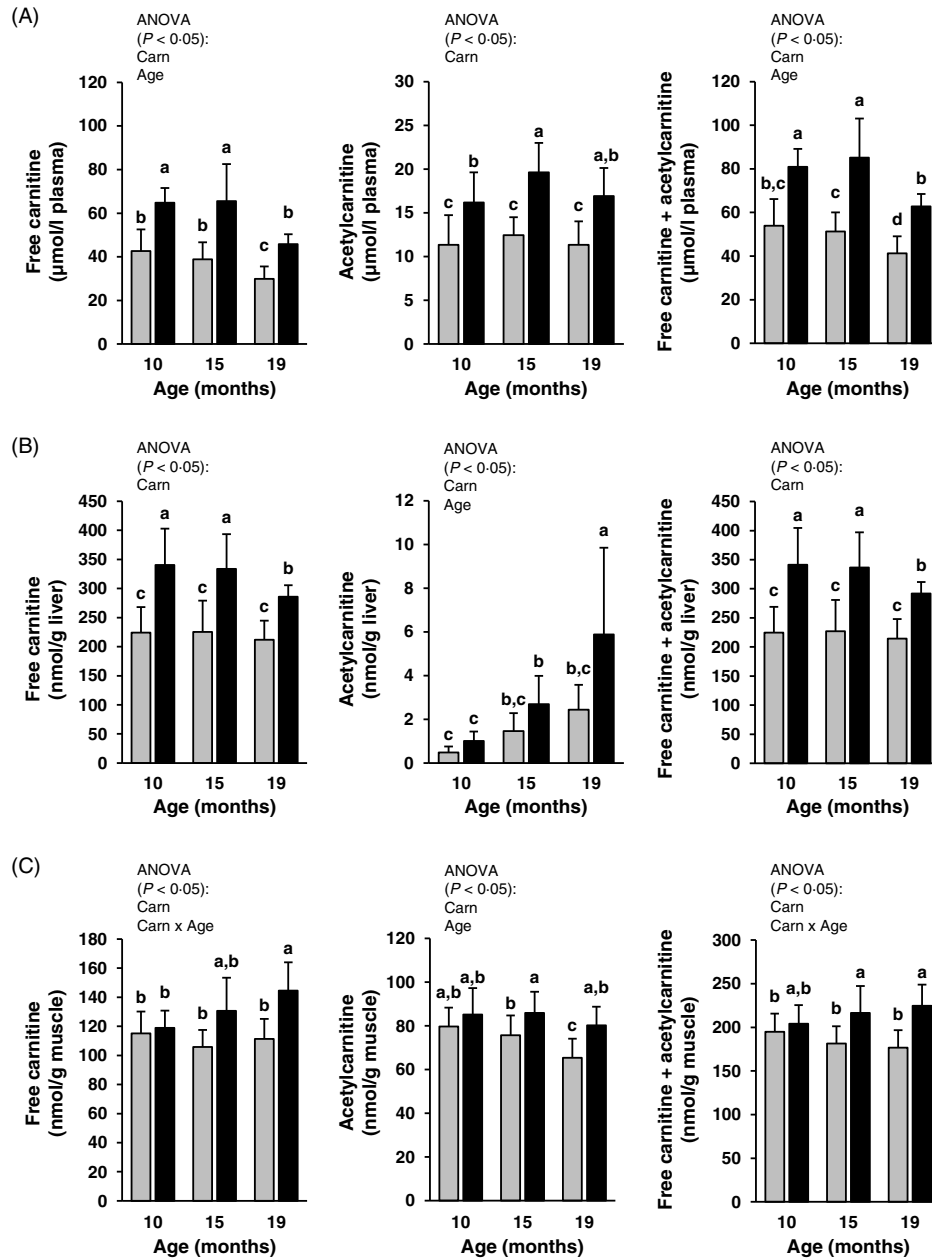
Fig. 1. Concentrations of free carnitine, acetylcarnitine and the sum of free carnitine and acetylcarnitine in plasma (A), liver (B) and skeletal muscle (C) of mice at 10, 15 and 19 months of age fed a control diet (▫) or a diet supplemented with 1000 mg/kg diet L-carnitine (■) from 3 weeks to 19 months of age. Bars represent mean values and standard deviations for n 10 mice/group. [a,b,c,d] Mean values with unlike letters are significantly different ($P<0·05$).

Relative mRNA and protein levels of genes involved in stress signalling in the liver of mice at 19 months of age

Relative mRNA concentrations of genes regulated by important stress signalling pathways (ER stress/unfolded protein response, NF-κB, nuclear factor-E2 related factor 2) in the liver of mice at an age of 19 months were not different between the carnitine group and the control group (online Supplementary Table S4). Relative protein concentrations of glutathione peroxidase and heme oxygenase, which are regulated by NF-κB and nuclear factor-E2 related factor 2, respectively, in the liver of mice at an age of 19 months did not differ between the carnitine group and the control group (online Supplementary Fig. S3).

Identification of genes regulated by carnitine supplementation in skeletal muscle of mice at 19 months of age

A total of 447 genes with official gene symbols (171 with FC>1·2, 276 with FC<−1·2, $P<0·05$) were identified as differentially expressed in skeletal muscle of 19-month-old mice between the carnitine and the control group (online Supplementary Tables S5 and S6). The most strongly up- and down-regulated genes in the carnitine group compared with the control group were β-1,4-N-acetyl-galactosaminyl transferase 2 (B4galnt2) (+3·11-fold) and the ankyrin repeat domain 2 (Ankrd2) (−2·52-fold), respectively. Validation of microarray data by
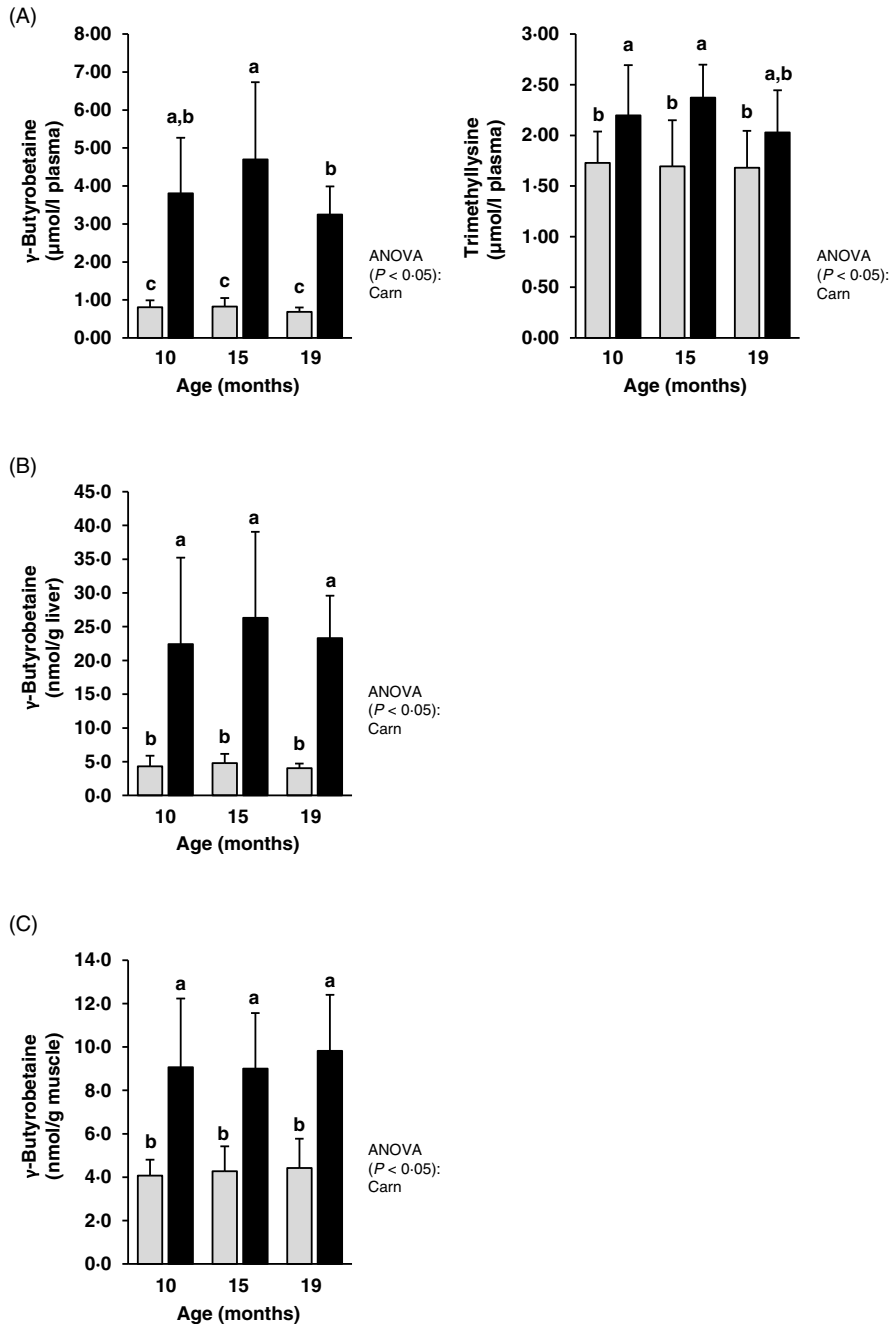
Fig. 2. Concentration of the carnitine precursor γ-butyrobetaine and trimethyllysine in plasma (A), liver (B) and skeletal muscle (C) of mice at 10, 15 and 19 months of age fed a control diet (▨) or a diet supplemented with 1000 mg/kg diet L-carnitine (■) from 3 weeks to 19 months of age. Bars represent mean values and standard deviations for *n* 10 mice/group. [a,b,c] Mean values with unlike letters are significantly different (*P*<0·05).

qPCR analyses of twelve differentially expressed genes showed that for all genes validated effect direction was the same between qPCR and microarray, but effect size (FC) differed more or less between qPCR and microarray (Table 3). Due to the different number of biological replicates (microarray: *n* 5, qPCR: *n* 10) and the different detection principle of transcript abundance between microarray and qPCR the FC and *P* values differed more or less. Statistical analysis revealed that five out of the twelve mRNA analysed by qPCR were regulated by carnitine supplementation at a significance level of *P*<0·05.

Identification of biological processes and molecular functions affected by carnitine supplementation in skeletal muscle of mice at 19 months of age

To identify biological processes and molecular functions affected by the genes regulated by long-term supplementation of carnitine in skeletal muscle of mice, GSEA was performed using GO biological process and molecular function terms. GSEA of the 171 up-regulated genes revealed that the top-enriched GO biological process terms (lowest enrichment

Table 3. Quantitative real-time PCR (qPCR) validation of microarray data for selected differentially expressed transcripts (fold change (FC)>1·2 or <−1·2, $P<0\cdot05$) in skeletal muscle of 19-month-old mice fed a control diet or a diet supplemented with 1000 mg/kg diet ʟ-carnitine from 3 weeks to 19 months of age*

Gene symbol	FC carnitine v. control		P
	Microarray	qPCR	qPCR
B4galnt2	3·11	2·20	0·155
Perp	2·16	1·92	0·032
Pld5	2·16	2·41	0·027
Ostn	2·09	1·51	0·113
Gamt	1·53	2·66	0·049
Tppp3	1·48	1·75	0·014
Ankrd2	−2·52	−2·08	0·036
Olfr142	−1·53	−1·20	0·470
Olfr618	−1·46	−1·14	0·669
Olfr1052	−1·43	−1·33	0·666
Olfr1356	−1·40	−1·28	0·520
Olfr427	−1·38	−1·23	0·612

B4galnt2, β-1,4-N-acetyl-galactosaminyltransferase 2; Perp, PERP, TP53 apoptosis effector; Pld5, phospholipase D family member 5; Ostn, osteocrin; Gamt, guanidino-acetate N-methyltransferase; Tppp3, tubulin polymerisation promoting protein family member 3; Ankrd2, ankyrin repeat domain 2; Olfr, olfactory receptor.
* The microarray FC was calculated from the signal log ratios as follows: $2^{\text{Signal log ratio}}$, if signal log ratio≥0 and $(-1) \times 2^{-(\text{Signal log ratio})}$, if signal log ratio<(0). Signal log ratios were calculated from five microarrays/group. The qPCR FC was calculated analogously from normalised $2^{-\Delta Ct}$ ratios. Normalised $2^{-\Delta Ct}$ expression was calculated from n 10 mice/group.

P value) were macromolecular complex assembly, homotypic cell–cell adhesion and cellular component biogenesis. The top-enriched GO molecular function terms (lowest enrichment P value) of the 171 up-regulated genes were peptidase activity, ubiquitin-like protein-specific protease activity and enzyme binding. All GO biological process and molecular function terms assigned to the up-regulated genes with an enrichment P value <0·01 are shown in online Supplementary Table S7.

GSEA of the 276 down-regulated genes showed that the top-enriched biological process terms (enrichment P value <0·01) were in decreasing order sensory perception of smell, sensory perception of chemical stimulus and sensory perception. The most enriched GO molecular function terms (enrichment P value <0·01) were in decreasing order G-protein-coupled receptor activity, olfactory receptor activity and transmembrane receptor activity. All GO biological process and molecular function terms assigned to the down-regulated genes with an enrichment P value <0·01 are shown in online Supplementary Table S8.

Discussion

An important finding of the present study is that lifelong carnitine supplementation at a dose comparable with that used in clinical trials is effective in preventing the age-dependent impairment of plasma carnitine status. In addition, a key finding of the present study is that feeding of supplemental carnitine during whole life generally leads to an elevated carnitine status in plasma and tissues of mice up to advanced age compared with age-matched non-supplemented control mice. This clearly indicates that the animal's plasma and tissue carnitine status profits from lifelong carnitine supplementation. With regard to the effect of carnitine

supplementation on carnitine status, some tissue specificities were found, such as supplemental dietary carnitine increased the concentration of the sum of free carnitine and acetylcarnitine in plasma and liver markedly stronger than in skeletal muscle. In addition, skeletal muscle carnitine status was improved by carnitine supplementation only at an age of 15 months and above. This relative 'resistance' of skeletal muscle carnitine content to even high doses of supplemental dietary carnitine is in agreement with findings from other studies in mice[37], rats[38,39] and humans[40,41] and has been attributed to the specific characteristics of skeletal muscle carnitine uptake which is mediated by OCTN2-transporting carnitine in a Na^+-dependent and saturable manner from plasma into skeletal muscle against a considerable concentration gradient (>100-fold)[42]. Due to the K_m of OCTN2 for carnitine (4·3 μM[43]), muscle OCTN2 is saturated even under normal blood levels of carnitine, and it is unlikely that increasing plasma carnitine availability increases muscle carnitine content. However, muscle carnitine content can be increased by 15–20 % by experimentally induced hypercarnitinaemia combined with intravenously infusing insulin or ingesting relatively large quantities of carbohydrates[44,45]. The stimulatory effect of insulin on skeletal muscle carnitine uptake has been explained by increasing sarcolemmal Na^+/K^+-ATPase activity and intracellular Na^+ flux, thereby increasing Na^+-carnitine cotransport via OCTN2[46]. Nonetheless, species differences exist with regard to the effect of exogenous carnitine on muscle carnitine content. In pigs, feeding the same dose of supplemental carnitine as in the present study for only 3–4 weeks increased the concentration of free carnitine and acetylcarnitine in skeletal muscle by greater than 2·5-fold[47,48]. Although the exact reason for these species differences is unknown, it could be speculated that pig muscle has a higher capacity for carnitine uptake from plasma due to higher expression level of Octn2 in skeletal muscle than in rodents and humans.

As a reason for the impaired plasma carnitine status of mice at advanced age, a decreased intestinal absorption and renal reabsorption of carnitine might come into question. Both intestinal absorption of dietary carnitine and renal reabsorption of filtered carnitine are dependent on the activity of the aforementioned OCTN2 present in intestinal epithelial cells[49] and renal tubular cells[50]. Interestingly, a recent study clearly showed that Na^+-dependent carnitine uptake in renal brush-border membrane vesicles isolated from whole kidney is significantly decreased in 8-month-old rats compared with 2-month-old rats[51]. In another study, it was demonstrated that mRNA expression of Octn2 in jejunum and ileum is lower in 6-month-old rats than in 1-month-old rats[52]. In addition, in humans a down-regulation of Octn2 was observed in mononuclear cells of healthy elderly compared with healthy adults[53]. These findings from rats and humans are indicative of an age-induced impairment of Na^+-dependent carnitine uptake. Despite direct proof in mice is lacking, an age-induced decline of intestinal carnitine absorption and renal carnitine reabsorption may therefore account for the decreased plasma carnitine status in mice at advanced age. Apart from intestinal absorption and renal reabsorption, plasma carnitine status is influenced by endogenous synthesis of carnitine in certain tissues. Endogenous synthesis of carnitine occurs via stepwise,

enzyme-catalysed conversion of free trimethyllysine, the initial carnitine precursor, into γ-BB, the ultimate precursor of carnitine, which is finally hydroxylated by γ-BB hydroxylase 1 (encoded by *Bbox1*) into carnitine. Because the activity of γ-BB hydroxylase 1 is restricted to liver and kidney in most mammals[54], these two organs play a central role in endogenous carnitine biosynthesis. However, non-hepatic and non-renal tissues, like skeletal muscles, also contribute to carnitine biosynthesis through providing γ-BB. In the present study, the plasma concentration of free trimethyllysine, whose availability is considered to be limiting for carnitine biosynthesis[55], and plasma and tissue concentrations of γ-BB in control mice were not different between the different age groups. This indicates that carnitine biosynthesis is probably not impaired at advanced age compared with adult age. In the context of γ-BB, a further interesting observation was that its plasma and tissue concentration was generally markedly higher in carnitine-supplemented mice than in control mice. Elevated plasma levels of γ-BB in carnitine-supplemented subjects have been also reported from others[56,57] and have been suggested to be the result of inhibition of γ-BB hydroxylase 1 activity by carnitine[58], thereby resulting in the accumulation of γ-BB. However, it has also been shown that gut microbial metabolism of dietary carnitine produces quantitatively significant amounts of γ-BB in the gut, particularly in the small intestine, of mice[59] and rats[60,61], and γ-BB is a good substrate for the carnitine transporter OCTN2[43], thereby facilitating the intestinal absorption of γ-BB. In line with this, Koeth *et al.*[59] demonstrated that plasma levels of γ-BB in carnitine-supplemented mice showed a 100-fold increase compared with control mice receiving no carnitine. Furthermore, suppression of gut microbiota with an oral antibiotic cocktail eliminated plasma levels of γ-BB in carnitine-supplemented mice[59], which strongly suggests that the majority of plasma γ-BB in carnitine-supplemented mice originates from gut microbial metabolism of dietary carnitine and not from inhibition of enzymes involved in carnitine synthesis.

In order to investigate the implications of carnitine supplementation-induced improvement of carnitine status in the liver of mice at advanced age, we studied the effect on key metabolic and stress signalling pathways in the liver by determining mRNA and protein levels of genes involved in these pathways. Hepatic mRNA levels of genes involved in lipid synthesis, which are under the control of SREBP, and of most genes involved in lipid catabolism, which are regulated by PPARα, were not different between 19-month-old mice of the control group and the carnitine group. An exception was that the hepatic mRNA level of *Aldh9a1* was elevated, and that of *Bbox1* tended to be elevated in the carnitine group compared with the control group. Despite being speculative, it might be that increased mRNA levels of genes involved in carnitine biosynthesis (*Aldh9a1* and *Bbox1*) is a compensatory effect to the known inhibition of γ-BB dioxygenase activity by carnitine[58]. However, at the protein level, no increase in the expression of ALDH9A1, BBOX1 and OCTN2 was seen in the liver of the carnitine group. Thus, taken together, we suggest that key lipid metabolic pathways regulated by SREBP and PPARα in the liver are not affected by carnitine supplementation in

mice of advanced age. In line with this, the concentrations of TAG and cholesterol in liver and plasma did not differ between mice of the two groups at 19 months of age. This clearly indicated that lifelong carnitine supplementation has neither beneficial nor detrimental effects on hepatic lipid metabolism. Apart from key metabolic pathways in the liver, we also considered possible implications of the improvement of carnitine status in the liver of carnitine-supplemented mice at advanced age on hepatic stress signalling and stress adaptation pathways. Our investigations at the mRNA and protein level revealed no effect of carnitine supplementation on expression of genes involved in important stress signalling and stress adaptation pathways in the liver of 19-month-old mice between the control and the carnitine group. Genes involved in ER stress/unfolded protein response signalling and genes regulated by NF-κB and nuclear factor-E2 related factor 2, which are known to be induced in response to ER stress-induced unfolded protein response in order to improve cellular protective mechanisms to cope with ER stress[62], are very sensitive to different kinds of stressors including metabolic, inflammatory, prooxidative and microbial signals[63]. Despite acute ER stress induces several homeostatic mechanisms to preserve ER function and cell function, chronic induction of ER stress has been shown to play a pathophysiological role in a number of diseases, such as fatty liver, obesity, diabetes and many others[64]. Thus, the unaltered expression of ER stress-sensitive genes in the liver of 19-month-old mice suggests that lifelong carnitine supplementation does not represent a stressful condition to the animal.

Apart from the liver, we also studied possible implications of carnitine supplementation-induced improvement of carnitine status in skeletal muscle of mice at advanced age by genome-wide transcript profiling. Despite the large number of genes identified as differentially regulated by supplemental carnitine, which is partially attributed to the mild filter settings applied, the very low number of genes regulated more strongly (e.g. only ten and six genes were regulated >1·5-fold and <–1·5-fold, respectively) indicates that the influence of lifelong carnitine supplementation on the skeletal muscle transcriptome of 19-month-old mice is very moderate. The most strongly up-regulated gene in skeletal muscle of the carnitine group was *B4galnt2* which encodes an acetylgalactosaminyl transferase that is confined in expression to specific specialised membrane domains in skeletal muscle[65]. Interestingly, overexpression of *B4galnt2* in skeletal muscle was reported to inhibit the development of muscle pathology in a mouse model of congenital form of muscular dystrophy[66]. By contrast, the most strongly down-regulated gene in skeletal muscle of the carnitine group was *Ankrd2* encoding the most abundant isoform of muscle ankyrin repeat proteins, which are stress-responsive proteins transducing different stress signals such as stretch, denervation and exercise in skeletal muscle[67–69]. Although the true biological meaning of carnitine-induced regulation *B4galnt2* and *Ankrd2* in skeletal muscle of mice is hard to interpret, it indicates that dietary carnitine has the potential to modulate pathophysiological processes and stress responses in skeletal muscle. To further interpret the biological meaning of carnitine supplementation-induced changes in the skeletal muscle transcriptome of 19-month-old mice, GSEA was performed with

the differentially regulated genes. Noteworthy, GSEA for the down-regulated genes showed an involvement of the encoded gene products in sensory perception of smell and G-protein coupled receptor signalling pathway. The enrichment of these biological processes is a highly interesting observation which is explained by the fact that the list of down-regulated genes contained a large number of genes (>60) encoding olfactory receptors (*Olfr* isoforms) and vomeronasal 1 and 2 receptors (*Vmn1r* and *Vmn2r* isoforms). Receptors of the *Olfr*, *Vmn1r* and *Vmn2r* gene families comprise the largest family of G protein-coupled receptors, which recognise and sense volatile chemicals (i.e. odorants) serving as exogenous ligands in order to facilitate perception of smell in the brain[70]. Due to this, these receptors are extensively expressed in the nasal olfactory epithelium, specifically in the cilia of olfactory sensory neurons. However, whole-genome sequencing studies convincingly demonstrated that these receptors are also ectopically expressed in non-chemosensory tissues like testis, kidney and skeletal muscle[71], where these receptors are also functional and activated by endogenous ligands. Interestingly, recent evidence indicates that skeletal muscle *Olfr* isoforms play a role in skeletal muscle development, regeneration and even muscular dystrophy[72,73]. Thus, although the biological significance of the results from GSEA should also be interpreted cautiously considering the overall very moderate regulation of genes by carnitine supplementation in mice, the regulation of more than sixty genes encoding chemosensory receptors by carnitine supplementation in skeletal muscle of 19-month-old mice strongly suggests that supplemental carnitine has the potential to modulate processes involved in muscle physiology and pathophysiology. Future studies are warranted to clarify the biological consequences of regulation of a large set of genes encoding chemosensory receptors in skeletal muscle by dietary carnitine.

The absence of detrimental effects of carnitine supplementation in our study is in strong contrast to two recent studies in mice demonstrating an impairment of cardiovascular health and liver function by carnitine supplementation[20,21]. However, the findings from the two mice studies are also in contrast to the vast majority of clinical studies which did not observe any adverse effects of carnitine supplementation[8,22,23]. An important difference between the studies of Koeth *et al.*[20] and Lu *et al.*[21] and clinical studies is the extremely high dose of carnitine per kg BW which was approximately 40–100 times higher in the mice studies than in clinical studies. Considering that in our study, the almost lifelong administration of carnitine at a dose which was similar to that used in clinical studies did not provoke adverse health effects, we postulate that the adverse effects reported recently are the result of the high dose of carnitine administered. It is likely that excessive formation of trimethylamine-*N*-oxide through gut microbiota-dependent conversion of carnitine into trimethylamine, which is subsequently converted to trimethylamine-*N*-oxide by hepatic flavin monooxygenase, is responsible for the adverse effects of supplemental carnitine, because multiple detrimental effects of trimethylamine-*N*-oxide have been identified such as inhibition of reverse cholesterol transport and impairment of hepatic and intestinal and sterol metabolism[74,75].

In conclusion, lifelong carnitine supplementation in mice prevents an age-dependent impairment of plasma carnitine status and improves the carnitine status of liver and skeletal muscle up to advanced age. While the improvement of tissue carnitine status in mice at advanced age was not accompanied by any detrimental changes in hepatic lipid metabolism and stress signalling pathways, moderate changes in skeletal muscle transcriptome might be indicative of the potential of carnitine to modulate muscle physiological processes. Based on the present observations, safety concerns associated with long-term supplementation of carnitine can be considered as unfounded, at least at doses used in clinical trials and with regard to the parameters investigated. This assumption is supported by the fact that none of the carnitine supplementation studies in humans reported any adverse effects up to daily doses of 6 g carnitine per d.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114519000709

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