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The influence of a chronic L-carnitine administration on the plasma metabolome of male Fischer 344 rats*

Christoph H. Weinert[1, †], Michael T. Empl[2, †], Ralf Krüger[3, †], Lara Frommherz[1], Björn Egert[1], Pablo Steinberg[2], Sabine E. Kulling[1]

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† These authors contributed equally to this study

[1] Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Department of Safety and Quality of Fruit and Vegetables, Haid-und-Neu-Str. 9, 76131 Karlsruhe, Germany

[2] Institute for Food Toxicology and Analytical Chemistry, University of Veterinary Medicine Hanover, Bischofsholer Damm 15, 30173 Hanover, Germany

[3] Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Department of Physiology and Biochemistry of Nutrition, Haid-und-Neu-Str. 9, 76131 Karlsruhe, Germany

Corresponding author: Dr. Christoph H. Weinert, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Department of Safety and Quality of Fruit and Vegetables, Haid-und-Neu-Str. 9, 76131 Karlsruhe, Germany. E-mail: christoph.weinert@mri.bund.de; Telephone: +49 (0)721 6625 526; Fax: +49 (0)721 6625 453

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Abstract

Scope: L-carnitine has been advertised as a fat-lowering and performance-enhancing supplement, although scientific evidence for its effectiveness is lacking. The uptake of about 1-2 g of L-carnitine per day may result in the formation of metabolites like trimethylamine-*N*-oxide (TMAO), which in turn may be converted to potential carcinogens or promote the development of cardiovascular diseases.

Methods and results: To assess whether an L-carnitine supplementation changes overall metabolism or causes the formation of previously unknown metabolites, we analyzed plasma samples from Fischer 344 rats originating from a previous study [2] using a multi-platform metabolomics approach comprising LC-MS/MS and GC×GC-MS methods. Despite an intake of up to 352 mg L-carnitine/kg body weight/day for one year, plasma concentrations of only 29 out of 359 metabolites were significantly influenced, the induced concentration changes being often comparatively small. Nevertheless, a clear dose-response relationship and a substantial concentration increase were observed for TMAO, i.e. a tenfold higher TMAO level was measured in the high-dose group when compared to the control (2.5 µM vs. 25.0 µM).

Conclusion: Although L-carnitine supplementation did not cause large changes in the plasma metabolome, a higher risk for cardiovascular disease due to chronically elevated TMAO plasma concentrations cannot be excluded.

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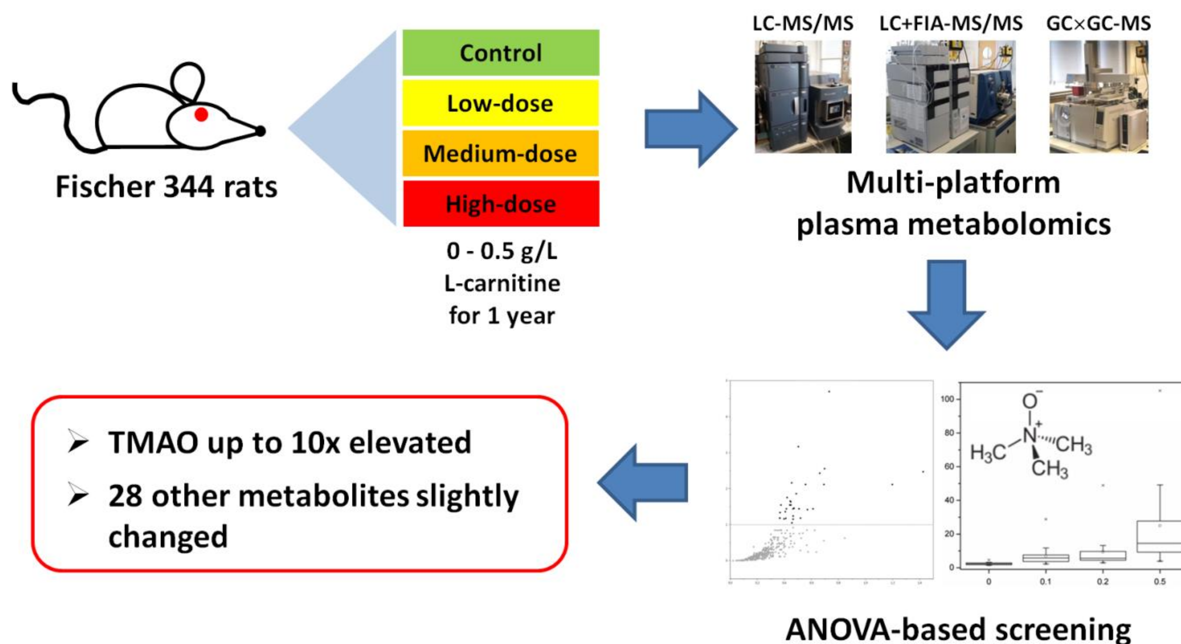
This study investigated if a chronic oral supplementation of L-carnitine causes metabolic changes in the plasma metabolite profile. Fischer 344 rats were supplemented with 0 to 0.5 g/L L-carnitine per day for one year. Plasma samples were analyzed with three different analytical platforms. Concentrations of 29 out of 359 reproducibly quantified metabolites were significantly changed by L-carnitine, but most of them only slightly. However, plasma TMAO increased up to tenfold.



1. Introduction

L-carnitine plays a major role in the degradation of long- and medium-chain fatty acids by enabling the transport of coenzyme A-conjugated (i.e., activated) fatty acids across mitochondrial membranes into the organelle's inner compartment, where they are degraded via β-oxidation [3]. Because of this, the consumption of L-carnitine as a dietary supplement has long been promoted as a fairly easy means to lower body fat and enhance athletic performance, although, up to now, there is no conclusive scientific proof that these claims are in fact true [4, 5]. Moreover, this perspective does not take into account that the L-carnitine concentration in the body is tightly regulated: Excess oral L-carnitine is only in part absorbed (bioavailability of < 20 %), while a large part of the absorbed portion is effectively eliminated from the body [6]. Consequently, high oral doses of this compound would remain unabsorbed in the gastrointestinal tract, where they can be transformed or degraded to different metabolites such as trimethylamine (TMA), trimethylamine-*N*-oxide (TMAO) or γ-

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butyrobetaine by enteric bacteria or flavin-containing monooxygenases (FMO) in the liver [7-9]. More specifically, TMA produced by the gut microbiota is converted to TMAO by FMO isotype 3 (FMO3), an hepatic enzyme whose malfunction in case of a mutation might lead to the onset of trimethylaminuria (reviewed in [10]).

In general, L-carnitine itself is not considered to be harmful, but some L-carnitine metabolites may cause adverse effects. On the one hand, recent studies suggest that TMAO may promote atherosclerosis [9, 11]. On the other hand, it has been hypothesized that TMA and other L-carnitine metabolites could, under certain conditions, potentially be transformed to the carcinogenic *N*-nitrosodimethylamine [2, 12]. In the former study, Fischer 344 (F-344) rats received up to 0.5 g/l L-carnitine via drinking water for one year, however without developing preneoplastic, atherosclerotic or other lesions related to the L-carnitine treatment [2].

With this in mind and due to its important involvement in lipid metabolism as well as the potential health concerns emanating from its metabolites, we now examined if chronically administered L-carnitine leads to any other (metabolic) alterations in F-344 rats. Specifically, we chose the plasma metabolome as endpoint to investigate whether the chronic L-carnitine supplementation caused any biologically relevant changes. The analysis was performed by using targeted and untargeted metabolomics methods (LC-MS/MS and GC×GC-qMS) on plasma samples obtained from the above-mentioned study [2].

Materials and Methods

2.1. Chemicals

Methanol (GC grade) was purchased from Merck and acetonitrile (LC-MS grade) from VWR (both Darmstadt, Germany). *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) with 1 % trimethylchlorosilane (TMCS) was supplied by Macherey-Nagel (Düren, Germany) and *O*-

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methoxylamine hydrochloride was purchased from Chemos (Regenstauf, Germany). Pyridine, ammonium formate (HPLC grade) and formic acid (LC-MS grade) were obtained from Sigma-Aldrich (Steinheim, Germany), while standards for L-carnitine, TMAO and structurally related metabolites as well as their deuterated isotopologues were acquired from TRC (Toronto, Canada) and supplied by Biozol (Eching, Germany). Internal standards used for GC×GC-qMS analysis were purchased from ABCR (Karlsruhe, Germany) and Sigma-Aldrich. All solvents and chemicals needed for the preparation of the Absolute IDQ™ p180 kit (Biocrates, Austria) were of analytical grade or of higher quality and obtained from Sigma-Aldrich and VWR.

2.2. Animal study

The animal study was performed as previously described [2]. Briefly, 80 male F-344 rats were divided into four groups consisting of 20 animals each and given either no ("control" or 0 g/L) or three different doses of L-carnitine (0.1, 0.2 and 0.5 g/L) via the drinking water during one year. These doses were chosen to mimic a low, medium and high consumption of L-carnitine as it is found in many commercially available products supplemented with this compound. As previously calculated [2], these dosages translate to an average daily L-carnitine uptake of approximately 70, 141 and 352 mg L-carnitine/kg body weight per rat or human equivalent doses (HED's) of 11, 23 and 57 mg/kg body weight, respectively. Further information on the laboratory animals as well as on animal husbandry can be found in the above-mentioned reference.

2.3. Sample collection

Blood samples were collected at the end of the above-mentioned one-year study (see section 2.2) in December 2013, when all 77 animals that completed the study were euthanized by decapitation under anesthesia after having fasted overnight. Their blood (approx. 1 mL/tube) was directly collected in EDTA-containing tubes (Sarstedt, Nümbrecht, Germany), which were then centrifuged at 16.060 rcf for 3 min. The supernatants (plasma) were immediately shock-frozen in liquid nitrogen,

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stored at -20 °C for four weeks and, after transfer to the analytical lab, at -80 °C until analysis. The GC×GC-qMS analysis as well as the metabolite profiling with the Absolute IDQ™ p180 kit (including the acylcarnitines) were performed in March/April 2014. The samples intended for the first targeted LC-MS analysis were stored for approximately 9-10 months before being processed and analyzed.

2.4. Targeted LC-MS determination of L-carnitine, TMAO and structurally related metabolites

L-carnitine, TMAO and structurally related metabolites were quantified by HILIC-MS/MS. Detection was performed by positive ESI using the MRM mode with two transitions per analyte, except for dimethylglycine (DMG) and sarcosine, which showed only one intense fragment. Deuterated internal standards were used for compensation of matrix effects. The sample preparation consisted of a protein precipitation and dilution with acetonitrile (1:10) and subsequent centrifugation before transfer to LC vials. Matrix-adapted calibrators and controls were produced by spiking of human plasma. For further method details see section 1 of the supplementary material.

2.5. LC-MS metabolite profiling using the Absolute IDQ™ p180 kit

Acylcarnitines, amino acids, biogenic amines, phosphatidylcholines and sphingomyelins were determined by using the Absolute IDQ™ p180 kit developed by Biocrates (Innsbruck, Austria). A detailed description of the preparation and quantification process can be found elsewhere [13]. A 20 µL plasma aliquot was used for each extraction. Amino acids and biogenic amines were determined by LC-MS (see section 2 of the supplementary material). Phosphatidylcholines and sphingomyelins were analyzed by flow injection analysis. QC samples included in the kit were injected ten times in between study samples. In addition, six replicates of study-specific QC samples (see section 2.3 for details) were evenly distributed amongst the study samples, extracted and injected as duplicates.

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2.6. Untargeted GC×GC-qMS metabolome analysis and data processing

Samples were prepared as described in section 3 of the supplementary material. Briefly, proteins were removed by precipitation with cold methanol and centrifugation. After evaporation of the supernatant, the dried samples were methoximated and trimethylsilylated. To enable quality control and to correct for drift and offset effects, four pooled quality control (QC) samples were prepared per day in the same way as the study samples. For GC×GC-MS analysis, the system described by Weinert et al. [14] was used with the following minor modifications: (1) Oven temperature was ramped from 90 °C to 280 °C using slopes of 2-5 °C/min, followed by a bake-out step at 320 °C; (2) A modulation period of 4.5 s was chosen. The GC×GC-qMS analysis comprised five measurement days and in total 121 runs, including 40 QC sample injections and five reagent controls. The study samples were analyzed in a randomized order and QC samples were injected after the daily reagent blank and after every fifth sample. Data processing comprised two main steps, which were previously described in detail by Egert et al. [15]. In addition, an analyte-specific QC-based correction of drift and batch effects was performed.

2.7. Evaluation of data quality

2.7.1. Targeted LC-MS analysis

Recoveries were determined to be 81-93 % (2 levels) by using deuterated analogues, since no analyte-free plasma was available. Matrix effects were small and matrix-driven signal changes were -10 % to +20 % depending on the analyte. Precision (intra-day, inter-day) was between 2 % and 8 % (2-3 levels), except for sarcosine (10-15 %). The bias (estimate for accuracy) was between -8 % and +10 % in comparison to the calculated spike values. Depending on the analyte, LOQs between 0.4 and 9.4 µM were achieved (criterion: max. 25 % relative standard deviation [RSD]), and the linear range exceeded two orders of magnitude for most analytes. Stability in plasma was also examined, and the maximum deviations were between -11 % and +19 %, again depending on the analyte and storage conditions.

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2.7.2. Absolute IDQ™ p180 kit

Results from the QC samples included in the kit and from the study-specific QC samples were used to calculate the respective RSD and to determine the intra-day precision for each analyte within the acquisition batch. Analytes with a RSD of more than 25 % (based on the study-specific QC samples) and with more than 25 % of the values below LOD were not considered for further statistical evaluation.

2.7.3. Untargeted GC×GC-qMS analysis

After automatic processing, the quality of the GC×GC-qMS data set was evaluated in three steps: (1) Selection of reliable internal standards for general quality control; (2) Examination of reliability of the measurement results of the QC and study samples using the selected internal standards; (3) Qualitative and quantitative assessment of analyte clusters. In a first step, the intra-day and inter-day (overall) precision of the internal standards in the QC samples was found to be good to excellent (RSD ≤ 15 %), only the derivatives of *N*-methyl-L-serine exceeding this limit. In a following step, the integrity of the QC and study sample runs was assessed by calculating for each sample the mean relative deviation of the signal intensities of the reliable internal standards from the daily median of all samples. While all QC samples remained within the acceptance boundaries of 85-115 %, one study sample had to be rejected. Finally, the analytes detectable in at least 75 % of the study samples were closely inspected in order to remove known artefacts and remaining sections of noise band not eliminated during denoising as well as analytes affected by coelution and analytes exceeding mean intra-day repeatability limits (RSD ≤ 20 %; in case of trace analytes and intra-day trends, an RSD of up to 30 % was accepted as described by Dunn et al. [16]). The “rare” analytes present in less than 75 % of the study samples were screened for consistent qualitative differences between the groups (detected/not detected).

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2.8. Data imputation and aggregation

After evaluation of analytical quality, data were prepared for statistical analysis. At first, missing data resulting from analyte concentrations below LOD were replaced by LOD/2 (Absolute IDQ™ p180 kit) or a peak area of 10000 (half of the peak area cut-off used for peak integration; GC×GC-qMS analysis). In case of the targeted LC-MS method, only five sarcosine values were slightly below the LOQ. However, these values were not replaced in order to avoid a biased alteration of the data. Subsequently, data of the different platforms were combined leading to a global data matrix containing 359 analytes, with a limited number of analytes being detected with two methods.

2.9. Statistical analysis

Statistical analysis was performed with the *Response Screening* platform of JMP® 11 (SAS Institute, Böblingen, Germany) using a three-step procedure (see supplemental Figure S1 for a scheme of the workflow). The main idea behind this approach was to identify the discriminating metabolites in typical metabolomics data sets (many metabolites detected in relatively few samples) in a reasonably fast and yet reliable manner, focusing on single metabolite responses rather than metabolite patterns. In the first screening step, the presumably discriminating analytes were selected with a common one-way analysis of variance (ANOVA) combined with a multiple testing correction according to Benjamini and Hochberg [17]. To account for possible violations of ANOVA assumptions (normal distribution and equal variances) at the screening stage, a comparatively liberal false discovery rate (FDR) p-value of < 0.1 was chosen as threshold. In a second step, the previously selected analytes were further investigated to assess if ANOVA assumptions were met and, consequently, which downstream testing procedures should be used. Finally, analytes were categorized accordingly and the final ANOVAs and post-hoc tests were performed.

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Results and Discussion

3.1. Coverage of plasma metabolome

The resulting numbers of the analytes meeting the data quality criteria (see section 2.7) are provided in Table 1. Concerning the GC×GC-MS data set, no consistent qualitative differences between the groups were observed among the clusters detected in less than 75 % of the study samples. The analytes identified by library matching are listed in Table S1 of the supplement. The analytes that could be reliably quantified using the Absolute IDQ™ p180 kit are listed in the supplementary Tables S2-A and S2-B.

3.2. Effect of L-carnitine supplementation on plasma metabolite concentrations

An ANOVA-based two-step approach (see section2.9 and Figure S1) was used to identify metabolites whose plasma levels were influenced by the L-carnitine supplementation. With the help of the initial screening ANOVA, 32 of the 359 entities in the global data matrix were selected as potentially discriminating metabolites (FDR p-value < 0.1, i.e. FDR LogWorth or $-\log_{10}$(FDR p-value) < 1; see Figure 1). Subsequently, in the case of 29 metabolites, the existence of a significant between-group difference was substantiated by more conservative testing procedures (Table 2).

3.2.1. Effect on the L-carnitine plasma level

Although most of the plasma metabolites did not show any dependence on the L-carnitine supplementation, changes in the concentration of L-carnitine and its direct and structurally related metabolites as well as a number of other endogenous compounds were significant (Figure 1 and Table 2). The daily ingestion of 70-352 mg L-carnitine/kg body weight [2] led to a moderate but dose-dependent increase (+26 % in the 0.5 g/L group) of L-carnitine in the plasma of the rats, suggesting that renal reabsorption was almost saturated and the excretion rate therefore relatively high. This hypothesis is supported by data from a study performed by Mancinelli et al. [18] using the isolated perfused rat kidney, in which supraphysiological levels of L-carnitine and acetyl-L-carnitine led to a

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decrease of the tubular reabsorption rate. Moreover, a similar phenomenon was described in a study in humans, in which a repeated oral L-carnitine supplementation resulted in a continuously ("steady state-like") higher plasma concentration, while the urinary excretion rate was approximately threefold higher in carnitine-supplemented individuals [6]. Nevertheless, even the highest plasma L-carnitine concentration measured in the present study (67.8 ± 10.3 µmol/L in the 0.5 % group; Figure 2) is still in the physiological range described for humans as well as rats (10-80 µmol/L; see [19]), supporting the fact that carnitine homeostasis is closely controlled by efficient removal of excess L-carnitine from the body [6, 7]. Remarkably, the plasma L-carnitine concentration increase did not result in any change in plasma acylcarnitine concentrations (see Table 2 and Table S2-A), although an increase was described in rats fasting for 24 h or longer [20, 21].

The basal plasma concentrations of L-carnitine in the fasting control rats (53.9 ± 6.9 µmol/L; Figure 2) mostly resemble the concentrations of free/total L-carnitine determined in other studies conducted with healthy male F-344 rats [22, 23] but may differ between rat strains: While lower baseline (free) L-carnitine levels are usually observed in healthy male Sprague-Dawley rats (approx. 20-50 µmol/L [24-27]), plasma concentrations in healthy male Wistar rats seem to be more in line with values described in Fischer 344 rats (≈45-85 µmol/L [28]). Although a modest rise in the plasma L-carnitine concentration after an oral supplementation, as shown in the present study (Figure 2), was expected and known from several other studies using rats [23-25, 27], an actual quantitative comparison with these and other studies proves to be difficult due to major differences in L-carnitine administration and dosing, timing and length of treatment, rat strain and age as well as in the chosen measurement endpoint (e.g. L-carnitine concentrations in plasma vs. serum/other tissues).

3.2.2 Effect on the plasma levels of metabolites related to L-carnitine

In contrast to the slight increase of the L-carnitine plasma concentration after supplementation, an about tenfold increase of the plasma TMAO level (median: sixfold increase) was observed at the end

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of the study (control [0 g/L] group: 2.5 ± 0.7 µmol/L; high-dose [0.5 g/L] group: 25.0 ± 25.9 µmol/L; Figure 2). This finding was expected, as TMAO production is directly related to bacterial L-carnitine metabolism in the gut of the rat as well as in man [29-31]. Of course, when extrapolating these data to human subjects, one has to keep in mind that certain factors influencing TMAO production differ between rodents and humans (e.g. composition of the gut microbiota or FMO3 activity; reviewed by [32]). The high variation in TMAO concentrations in the high-dose group (Figure 2) is also remarkable. Whether this is related, for example, to differences between animals in the composition of the gut microbiota or in FMO expression can only be speculated and not substantiated based on the present data. Nevertheless, it is known that the extent of TMA formation in the gut from precursors such as L-carnitine or choline strongly depends on the composition of the gut microbiota [9, 33] and variations in its composition and consequently TMAO formation might be due to subtle environmental or housing differences. It should also be noted that, independently of the treatment, almost all animals used in the present study suffered from a suppurative and necrotizing hepatitis [2], a condition which might have influenced the metabolizing function of the liver and especially that of FMO to different degrees [34]. In addition, FMO expression levels depend on factors such as substrate availability and turnover. A third possible explanation could involve different renal excretion rates, as it is well-known that renal function is closely related to TMAO levels in blood and urine [12, 35, 36]. As in the case of the baseline plasma L-carnitine levels (see above), there also seems to be a certain variance regarding TMAO plasma concentrations between different rat strains. While Ufnal et al. [37] report a baseline plasma concentration of ≈0.57 µmol/L in twelve-week-old Sprague Dawley rats, the values presented herein exceed this concentration almost fivefold. However, it has to be kept in mind that the animals used in the present study were much older at the time of sampling and that there might be differences in the fed diets regarding TMAO precursor content (e.g. choline).

TMAO has recently been linked to the development of cardiovascular diseases [9, 11, 38]. Even though the chronic L-carnitine supplementation increased TMAO production in F-344 rats (Figure 2),

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no atherosclerotic lesions were found in the treated animals [2]. This is most probably due to the fact that F-344 rats are not prone to develop atherosclerosis [39]. This rat strain was nevertheless chosen because it was found to be appropriate to clarify if a long-term L-carnitine supplementation promotes the formation of preneoplastic lesions in the colon. In the light of the current discussion on TMAO as a risk factor for cardiovascular diseases, possible negative effects of a long-term, high-dose L-carnitine supplementation and subsequent high TMAO plasma levels on cardiovascular health cannot be excluded. Thus, the elevated TMAO level is not only the most obvious change resulting from L-carnitine supplementation; it is probably also the most relevant effect with respect to possible health risks. However, the actual health hazard cannot be ultimately estimated, even though a link between elevated plasma TMAO levels and cardiovascular diseases has been established in suitable animal models as well as in humans [9, 38].

In contrast to the two compounds mentioned above, chronic L-carnitine supplementation resulted in a dose-dependent decrease in the plasma levels of betaine, DMG and sarcosine (-15 %, -17 %, and -46 % maximum fold change, respectively; Figure 2). Choline levels were also decreased (-24 %; Figure 2), albeit only significantly in groups 2 and 3 (0.1 and 0.2 g/L L-carnitine, respectively) but not in group 4 (0.5 g/L L-carnitine). Choline plays an important role in the synthesis of the neurotransmitter acetylcholine and membrane phospholipids (e.g. phosphatidylcholine and lysophosphatidylcholine). Betaine (an oxidation product of choline), DMG (a demethylation product of betaine) and sarcosine (a demethylation product of DMG) are, among other compounds, mainly involved in the one-carbon cycle as methyl group donors, thus contributing to maintain proper DNA methylation [40]. Aberrant DNA methylation has been associated with different diseases, including cancer [41]. Due to the rather small (although statistically significant) L-carnitine-related changes (15-24 %) in the plasma concentrations of choline, betaine and DMG (Figure 2), an impact on the one-carbon or another metabolic cycle of the rat seems improbable. However, in the case of sarcosine, the L-carnitine-associated relative decrease of the plasma concentration is rather substantial (-46 %, Figure 2), a fact which might be explained by sarcosine levels being generally

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rather low in all groups and only a factor 2-3 above the LOQ of the LC-MS method. Moreover, since no changes in the plasma glycine and serine as well as hippuric acid levels were detected [40, 42, 43], a direct connection with the one-carbon metabolism seems unlikely, although an analysis of tetrahydrofolate and methylenetetrahydrofolate plasma levels would shed more light on this unresolved issue. While the basal (control) free choline plasma concentrations measured in the present study (15.9 ± 4.2 µmol/L) were roughly comparable to those described in other studies using Sprague-Dawley [44, 45] as well as Wistar rats [46, 47], control plasma betaine levels (87.2 ± 9.1 µmol/L) were almost twice as low as reported elsewhere (186 ± 43 µmol/L in male Sprague-Dawley rats [48]). Again, it remains unknown whether this is due to the rat strain used in the present study or to other factors. In contrast, basal rat plasma concentrations of DMG (4.9 ± 0.8 µmol/L) and sarcosine (1.0 ± 0.3 µmol/L) have, to our knowledge, never been published before.

3.2.3 Effect on the global plasma metabolite profile

Apart from the six metabolites mentioned above, the plasma concentrations of several other plasma metabolites were influenced by the L-carnitine supplementation, many of them being nitrogen-containing compounds or lipids (Table 2). As in the case of the structurally related metabolites (see above), a higher L-carnitine load generally resulted in a decrease of the concentration of some metabolites (β-hydroxybutyric acid, xylose, allantoin, pentadecanoic acid and creatinine), thereby displaying a clear dose-effect relationship. On the other hand, a significant concentration reduction in the case of compounds like ribose, 2-ethyl-3-hydroxypropionic acid (tentatively identified), serotonin, glyceric acid, ornithine or alanine was only observed because the plasma concentration of only one group (mostly the control) differed, while the levels in the other groups remained approximately the same (Table 2). However, with the exception of a few selected compounds (serotonin, spermidine, 2-ethyl-3-hydroxypropionic acid, ribose and xylose; Table 2), the changes were rather small when compared to the control animals, again suggesting that a long-term L-

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carnitine supplementation probably does not cause major shifts in the analyzed part of the metabolome. It is difficult to deliver an explanation for the plasma level reduction of certain metabolites, as their physiological/metabolic pathways are only vaguely interconnected. Nevertheless, e.g. serotonin, spermidine and ribose metabolism are (distantly) interconnected through the one-carbon and folate cycle [49, 50]. This could explain the observed decrease in the plasma levels of these metabolites, as the levels of some other molecules (see above) involved in these pathways seem also to be affected by L-carnitine supplementation, although merely to a small extent.

In contrast, the metabolites whose plasma concentrations significantly increased were not as numerous and included only stearoyl-lysophosphatidylcholine, stearoyl-L-carnitine and an unknown amine (Table 2). The unknown amine had a retention index of 1874 (based on saturated fatty acid methyl esters), but unfortunately could not be further characterized because of its rather unspecific spectrum. Again, since the stearoyl-lysophosphatidylcholine and stearoyl-L-carnitine concentration increases were quite small (+12 % and +17 %, respectively), they are most probably not biologically relevant. However, especially the plasma level increase of stearoyl-lysophosphatidylcholine in the high-dose group points out to a possible involvement of the L-carnitine supplementation in atherosclerosis, possibly via TMAO production, as has been suggested e.g. by Koeth et al. [9]. Lysophospholipids originate from the phospholipase A_2 (PLA_2)-mediated cleavage of cell membrane phospholipids [51, 52] and have been suggested, together with $PLA_{2,}$ to be involved in atherosclerosis development [53-55]. Whether this stearoyl-lysophosphatidylcholine plasma level increase is related to an interference or an increase of L-carnitine with PLA_2 activity or another pathway affecting membrane lipid depletion/turnover (e.g. after cellular damage [56]) remains purely speculative at the moment and would need to be confirmed in further experiments. However, it has to be mentioned that stearoyl-lysophosphatidylcholine has been described as an inhibitor of rat plasma PLA_2 [56], which somehow contradicts this assumption.

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The multi-platform approach used in this study covered several classes of lipids (see supplemental Tables S1, S2-A and S2-B). Slight but significant changes were only observed for three different phosphatidylcholine classes, stearoyl-lysophosphatidylcholine, stearoyl-L-carnitine, octadecadienyl-L-carnitine, and pentadecanoic acid (between +12 % and -21 %, see Table 2), while the concentrations of all other lipids remained unchanged. Thus, based on the set of lipid species covered here, the L-carnitine supplementation seems to only exert a minor influence on the lipid profile in the plasma of F-344 rats. This finding is in line with the reports of Eder et al. [57] and Brandsch et al. [27] but in contrast to the results of Tanaka et al. [22]. Moreover, differing outcomes were observed in other animal species: a lipid-lowering effect of an L-carnitine supplementation was demonstrated for broiler chickens [58], rabbits [59] as well as infants and patients with hyperlipidemia or hyperlipoproteinemia (reviewed in [60]). In contrast, L-carnitine ultimately turned out to be ineffective for the treatment of dyslipidemia of hemodialysis patients [61] and did not change the lipid profile of healthy adults [62]. In summary, although L-carnitine may exhibit a lipid-lowering effect under certain circumstances, it is not justified to generalize this claim.

Plasma levels of β-hydroxybutyric acid decreased in a dose-dependent manner by up to 24 % (control group versus 0.5 g/L group). This may point out to a reduced rate of β-oxidation. Again, this finding is in contrast to the outcome of previous studies in rats [27] and humans [62].

Concluding remarks

The effect of a long-term oral supplementation of L-carnitine on the plasma metabolite profile of male F-344 rats was investigated in the present study using a combination of targeted and untargeted metabolomics methods. The L-carnitine treatment significantly altered the plasma levels of 29 out of a total of 359 genuine metabolites. In most cases, the observed mean differences between the groups were small (fold change ±25 % when compared to control animals in the case of 20 out of 29 metabolites) and therefore probably not relevant from a biological point of view, except for TMAO. In this case, on average, a tenfold higher concentration was observed in the high-dose

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group when compared to the control group (2.5 µM vs. 25.0 µM). Thus, although a chronic supplementation of L-carnitine in doses of up to 0.5 g/L did neither induce adverse effects in the colon and aorta/heart of F-344 rats [2] nor drastic changes in the overall plasma metabolome, it led to permanently and substantially elevated plasma TMAO levels.

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Author contributions

M.T.E. and P.S. designed and conducted the initial animal experiment [2]. C.H.W., R.K., and L.F. performed analytical measurements and data quality control. B.E. processed the GC×GC-MS raw data. C.H.W. carried out the statistical evaluations. C.H.W., M.T.E., R.K., L.F., P.S., and S.E.K. wrote or contributed to writing of the manuscript.

Conflict of Interest

The authors have no conflict of interest to declare.

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Figure captions

Figure 1: Identification of potentially discriminating metabolites using an ANOVA screening approach. While the majority of plasma metabolites showed no significant concentration difference (grey dots), 32 metabolites with an FDR *p*-value lower than 0.1 (black dots above the dashed line, which denotes the screening threshold of FDR LogWorth = $-\log_{10}$(FDR *p*-value) = 1) were selected for further investigation. See Table 2 for identity of numerated metabolites. Not numerated black dots designate metabolites whose initially assumed significant concentration difference finally turned out to be insignificant according to more conservative downstream testing procedures.

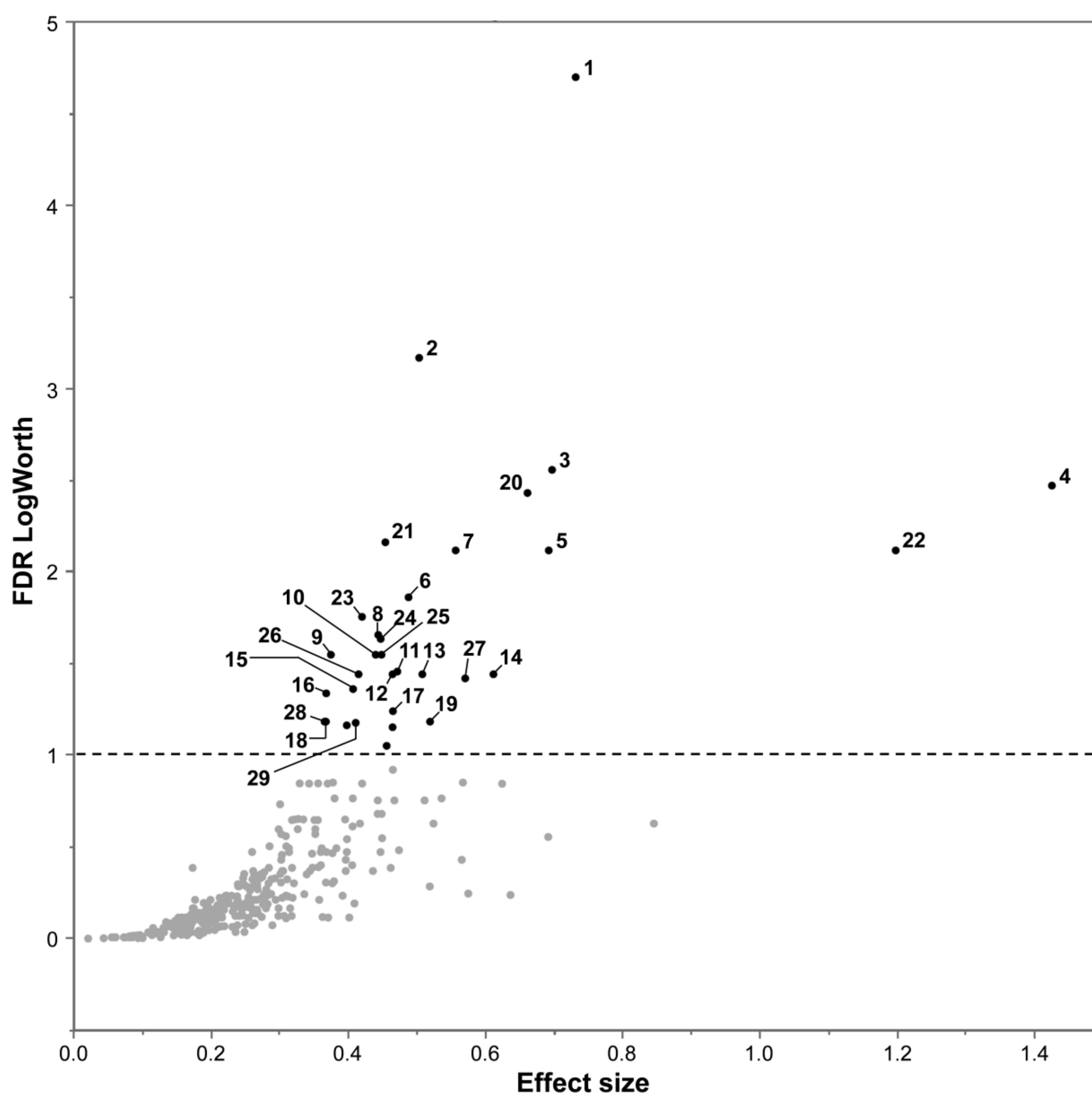

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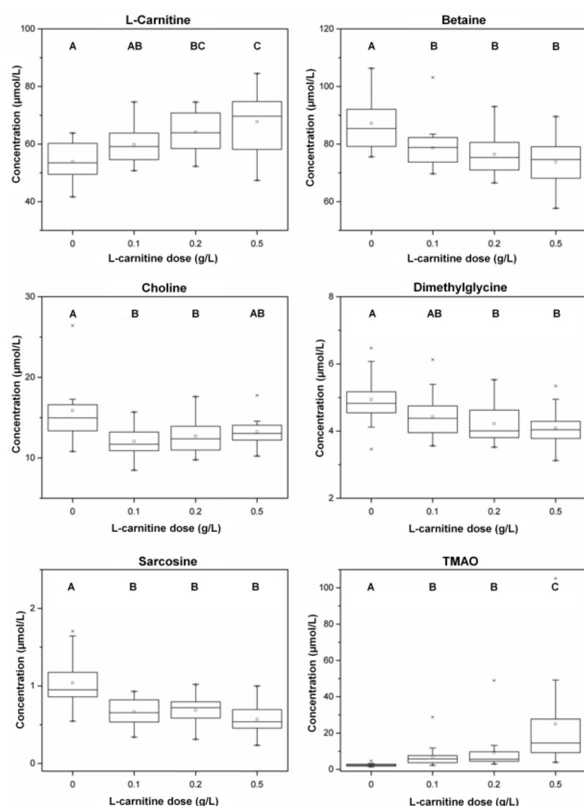
Figure 2: Box plots of the six metabolites covered by the targeted LC-MS method. Box: median, 25th + 75th percentile; square: mean; crosses: 1st + 99th percentile; whiskers: min + max. The letters A, B, C denote statistical significance ($p < 0.05$; differences between all four groups according to the Tukey-Kramer or Steel-Dwass test), whereby groups sharing the same letter do not significantly differ.



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Tables and table captions

Table 1: Numbers of frequent and reproducible analytes detected per platform.

	GC×GC-MS	Absolute IDQ™ p180 kit	Targeted LC-MS
Total number of detected analytes/features	656[a]	172[b]	6
Analytes detected in ≥ 75 % of the study samples	351	152	6
Analytes with sufficient intermediate precision	207	146	6

[a] Features detectable in at least 50 % of the animals belonging to one of the groups; [b] Metabolites detected in at least one sample (up to 188 metabolites can theoretically be quantified using this kit).

Table 2: Statistical evaluation of potentially discriminating metabolites (ordered by analytical platform and increasing FDR *p*-values).

No.	Analyte	1st ANOVA		Assumptions		2nd ANOVA			Homogeneous groups[k]				Fold change[l]
		FDR[d] *p*-	R^2	Normality	Homoscedasticity	Type of	*p*-value	Post hoc test	Control	0.1 g/L	0.2 g/L	0.5 g/L	
1	Sarcosine[a]	< 0.0001	0.39	yes	0.0365	Welch	< 0.0001	Steel-Dwass	A	B	B	B	0.54
2	L-carnitine[a]	0.0007	0.32	yes	0.1016	Standard	< 0.0001	Tukey-Kramer	A	AB	BC	C	1.26
3	Betaine[a]	0.0028	0.28	yes	0.5492	Standard	< 0.0001	Tukey-Kramer	A	B	B	B	0.85
4	TMAO[a]	0.0034	0.27	no	0.0011	Kruskal-Wallis	< 0.0001	Steel-Dwass	A	B	B	C	10.18
5	Choline[a]	0.0076	0.24	yes	0.0218	Welch	0.0066	Steel-Dwass	A	B	B	AB	0.76
6	Dimethylglycine[a]	0.0138	0.22	yes	0.5170	Standard	0.0004	Tukey-Kramer	A	AB	B	B	0.83
7	PC ae C42:0[b,e]	0.0076	0.24	yes	0.6293	Standard	0.0002	Tukey-Kramer	A	AB	B	B	0.84
8	L-carnitine[b]	0.0221	0.21	yes	0.0155	Welch	0.0002	Steel-Dwass	A	A	B	B	1.23
9	Octadecadienyl-L-carnitine[b]	0.0284	0.20	yes	0.7435	Standard	0.0012	Tukey-Kramer	A	B	B	B	0.83
10	lysoPC a C18:0[b,f]	0.0284	0.20	yes	0.0428	Welch	0.0174	Steel-Dwass	A	AB	A	B	1.12
11	Taurine[b]	0.0350	0.19	yes	0.6200	Standard	0.0017	Tukey-Kramer	A	B	B	B	0.80
12	Creatinine[b]	0.0362	0.19	yes	0.6687	Standard	0.0020	Tukey-Kramer	A	AB	B	B	0.91
13	Ornithine[b]	0.0362	0.19	yes	0.0389	Welch	0.0465	Steel-Dwass	A	AB	AB	B	0.75
14	Serotonin[b]	0.036	0.1	no	0.0027	Krusk	0.013	Steel-	A	AB	B	AB	0.34

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		2	8			al-Wallis	9	Dwass					
15	Alanine[b]	0.0436	0.18	yes	0.6964	Standard	0.0028	Tukey-Kramer	A	A	A	B	0.83
16	Spermidine[b]	0.0460	0.17	yes	0.6163	Standard	0.0031	Tukey-Kramer	A	A	AB	B	0.67
17	PC ae C34:3[b,g]	0.0575	0.17	yes	0.0556	Standard	0.0040	Tukey-Kramer	A	AB	B	B	0.84
18	Octadecanoyl-L-carnitine[b]	0.0657	0.16	yes	0.3106	Standard	0.0051	Tukey-Kramer	A	A	AB	B	1.17
19	PC aa C42:6[b,h]	0.0657	0.16	yes	0.4567	Standard	0.0048	Tukey-Kramer	A	AB	B	B	0.87
20	Glyceric acid TMS_3[c]	0.0037	0.27	yes	0.0530	Standard	0.0001	Tukey-Kramer	A	B	B	B	0.77
21	2-Ethyl-3-hydroxypropionic acid TMS_2[c,i]	0.0069	0.25	no	0.6769	Kruskal-Wallis	< 0.0001	Steel-Dwass	A	B	B	B	0.49
22	Ribose MeOX TMS_4[c]	0.0076	0.24	no	0.0002	Kruskal-Wallis	0.0150	Steel-Dwass	A	B	B	AB	0.31
23	Allantoin Derivative 2[c]	0.0176	0.22	yes	0.1273	Standard	0.0005	Tukey-Kramer	A	A	B	B	0.84
24	*β*-Hydroxybutyric acid TMS_2[c]	0.0232	0.21	yes	0.8776	Standard	0.0008	Tukey-Kramer	A	AB	B	B	0.76
25	Allantoin Derivative 1[c]	0.0284	0.20	yes	0.9451	Standard	0.0012	Tukey-Kramer	A	AB	B	B	0.75
26	Xylose MeOX-TMS_4[c]	0.0362	0.19	no	0.0026	Kruskal-Wallis	0.0048	Steel-Dwass	A	AB	AB	B	0.48
27	Pentadecanoic acid TMS[c]	0.0381	0.18	no	0.3766	Kruskal-Wallis	0.0058	Steel-Dwass	A	AB	B	B	0.79
28	Unknown amine[c]	0.0657	0.16	yes	0.0967	Standard	0.0050	Tukey-Kramer	A	AB	AB	B	1.30
29	Unknown[c]	0.0666	0.16	yes	0.0347	Welch	0.0381	Steel-Dwass	A	AB	B	AB	0.81

[a] Targeted LC-MS; [b] Absolute IDQ™ p180 kit; [c] Untargeted GC×GC-MS; [d] Calculated according to Benjamini and Hochberg (1995); [e] Phosphatidylcholine (acylated and alkylated), 42 C-atoms, no double-bonds; [f] Lyso-Phosphatidylcholine, acylated, one stearic acid side chain; [g] Phosphatidylcholine (acylated and alkylated), 34 C-atoms, three double-bonds; [h] Phosphatidylcholine (2 acyl residues), 42 C-atoms, six double-bonds; [i] Tentatively identified using the NIST2011 library; [j] *p*-value of Brown-Forsythe test;
[k] The letters A, B, C denote statistical significance: Groups sharing the same letter do not significantly differ; [l] Calculated for the two groups with the highest absolute difference in means.

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