Identification and regulation of the xenometabolite derivatives *cis*- and *trans*-3,4-methylene-heptanoylcarnitine in plasma and skeletal muscle of exercising humans

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Running head: Regulation of xenometabolite derivatives during exercise

Abstract

Little is known about xenometabolites in human metabolism, particularly under exercising conditions. Previously, an exercise-modifiable, likely xenometabolite derivative, *cis*-3,4-methylene-heptanoylcarnitine, was reported in human plasma. Here, we identified *trans*-3,4-methylene-heptanoylcarnitine, and its isomer *cis*-3,4-methylene-heptanoylcarnitine, in plasma and skeletal muscle tissue by liquid chromatography-mass spectrometry. We analyzed the regulation by exercise and the arterial-to-venous differences of these cyclopropane ring-containing carnitine esters over the hepato-splanchnic bed and the exercising and resting leg in plasma samples obtained in three separate studies from young, lean and healthy males. Compared to other medium-chain acylcarnitines, the plasma concentrations of the 3,4-methylene-heptanoylcarnitine isomers only marginally increased with exercise. Both isomers showed a >2-fold increase in the skeletal muscle tissue of the exercising leg; this may be due to the net effect of fatty acid oxidation in the exercising muscle and uptake from blood. The latter idea is supported by a >2-fold increased net uptake in the exercising leg only. Both isomers showed a constant release from the hepato-splanchnic bed, with an increased release of the *trans*-isomer after exercise. The isomers differ in their plasma concentration with a four times higher concentration of the *cis*-isomer. This is the first approach studying kinetics and uptake/release of xenolipid isomers from tissues under exercised conditions, supporting the hypothesis that hepatic metabolism of cyclopropane ring-containing fatty acids is one source of these acylcarnitines in plasma. The data also provide clear evidence for an exercise-dependent regulation of xenometabolite metabolism, opening perspectives for future studies about the physiological role of this largely unknown class of metabolites.

Keywords:

Xenolipids, exercise, arterial-to-venous difference, liver, microbiome

Abbreviations:

EIC, extracted ion chromatogram; tR, retention time; UHPLC-Q-TOF/MS, ultra high-performance liquid chromatography quadruple-time of flight mass spectrometry

Introduction

Long-chain acylcarnitines are the intermediates in the transport of long-chain acyl-CoA into the mitochondrial matrix, where the carnitine esters are reconverted to acyl-CoA for subsequent β-oxidation. The process is bidirectional, and acylcarnitines of long-, medium- and short-chain length formed in the mitochondrial matrix from acyl-CoA and carnitine can be exported and released into plasma (17, 23). A pronounced increase of circulating acylcarnitines levels is found in physiological and pathophysiological conditions (23), due to conditions of increased fatty acid oxidation during exercise (11) or fasting (21) or due to inherited enzymatic defects. On the other hand, elevated plasma levels are also a marker of metabolic disorders associated with diabetes (1, 18), obesity and insulin resistance (2, 3, 19, 20), and cardiovascular diseases (13). Almost all acyl-CoAs can be found as the respective carnitine esters in the circulation. Recent work expanded the knowledge on the complexity of acylcarnitine species by a comprehensive large-scale acylcarnitine identification resulting in a database of 758 acylcarnitines, having exact mass, retention time, and MS/MS information (26). Applying this database led to the reliable annotation of more than 240 acylcarnitine species in human plasma. The complexity is caused by the differences in chain length, grade of saturation and hydroxylation, but the database also contains several isomers with as-yet unclarified structure, origin and function.

A different regulation of isomers of a certain acylcarnitine in plasma may indicate differences in origin and (patho)physiological relevance. Along these lines, a pronounced increase in plasma medium-chain acylcarnitines is a significant feature in exercising humans (10, 14, 27). We recently reported a medium-chain acylcarnitine species showing an unusual time course, i.e. almost no increase in plasma, during and after 2 hour cycling exercise at 60% VO_2max (24). This metabolite was annotated as C8:1 acylcarnitine based on m/z and retention time. Later, an isomer of C8:1

acylcarnitine found in human plasma was identified as *cis*-3,4-methylene-heptanoylcarnitine (27). This acylcarnitine was postulated to be a xenometabolite derivative (27), because the acylgroup contains a cyclopropane ring which has only been described in fatty acids originating from bacteria and plants (4, 7). These fatty acids are found in various kind of foods like milk products and bovine meat (16), and may also be produced by gut bacteria (15). In any case, it has been postulated that the xenometabolites are absorbed and delivered via the portal vein and their partial oxidation results in the formation of the respective medium-chain acylcarnitine esters, which can be detected in plasma (27). In the present study, we analyzed the fluxes of the putative xenometabolite derivatives *cis*-3,4-methylene-heptanoylcarnitine and the newly identified *trans*-isomer over the hepato-splanchnic bed and the leg during exercise and recovery, to get more insights into the origins and metabolism of both compounds.

Methods and Materials

Blood samples

The blood samples used in this work were obtained from three different studies. In Study 1, peripheral plasma samples were collected before, immediately after 30 min of treadmill exercise at 105% individual anaerobic lactate threshold, and 120 min after the end of this exercise bout to assess the regulation of circulating medium-chain acylcarnitine levels by exercise. To investigate the fluxes of C8:1 acylcarnitine isomers over the hepato-splanchnic bed and over the resting and exercising leg, blood samples of two previously reported exercise studies (Study 2 and Study 3) were used (8, 9). All studies were executed in accordance with the Helsinki Declaration and were approved by the Ethical Committee of the University of Tuebingen (203/2014/BO2) (Study 1) or the Scientific Ethics Committee of the capital region of Denmark (Study 2 and Study 3). Subjects

participating in these three studies were not identical. All subjects provided written informed consent to participate. Blood was collected in EDTA containing tubes, placed on ice and immediately centrifuged, and aliquots were stored at -80^{o} C until ultra high-performance liquid chromatography quadruple-time of flight mass spectrometry (UHPLC-Q-TOF/MS) analysis.

In Study 1, the subjects were 20 young male endurance-trained runners (24.5 ± 3.9 years; 20.8 ±0.2 kg/m^2 BMI; 66.2 ± 1.7 ml/kg/min VO_2max).

In Study 2, ten healthy male subjects (age: 22.9 ± 0.8 years; BMI: 22.6 ± 0.5 kg/m^2) performed a 120 min-cycling exercise at 60% VO_2max in semi supine position after an overnight fast; subjects remained fasting throughout the whole experimental period (9). Briefly, catheters were inserted into the brachial artery of the non-dominant arm and into a liver vein via the right femoral vein. Blood samples were obtained in pairs from both catheters at each time point. Hepatic blood flow was measured by indocyanine green clearance (9). Samples obtained at baseline (0), 60, 120, 150, 180, 240, 300 and 360 min were used for analysis.

In Study 3, the effect of exercise on C8:1 acylcarnitine isomer fluxes over the leg was investigated by analyzing plasma samples from a one-legged knee extensor study where the exercising leg was compared to the other, resting leg (8). Briefly, after an overnight fast nine healthy male subjects (age: 20.9 ± 0.5 years; BMI: 22.6 ± 0.8 kg/m^2) performed continuous one-legged knee-extensor exercise for 120 min at 50% of maximum workload on a modified Krogh ergometer while the contra-lateral leg was resting. Catheters were inserted retrograde into both femoral veins as well as into one femoral artery. The subjects were fasted until 3 hours after the exercise bout. Blood flow was determined for both the resting and the exercising leg using ultrasound Doppler in three subjects and the mean value was used to calculate net skeletal muscle release/uptake of acylcarnitines for all subjects. Blood samples were drawn simultaneously from all three catheters

and samples obtained at baseline (0), 60, 120, 150, 180, 240, 300, and 360 min were used for analysis.

Skeletal muscle biopsies

In Study 3, percutaneous needle biopsies from the vastus lateralis muscle of the exercising and the resting leg were taken. Biopsies obtained at baseline (0 min), at the end of the exercise bout (120 min), as well as after 300 min were used for analysis.

Chemicals and reagents

HPLC or MS grade solvents acetonitrile, methanol (MeOH), chloroform ($CHCl_3$), anhydrous acetonitrile, chromium oxide (CrO_3), sulfuric acid (H_2SO_4), and diethyl ether were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Steinheim, Germany or Milwaukee, WI, USA). *Cis*-3-hepten-1-ol and *trans*-3-hepten-1-ol were purchased from Alfa Aesar (Tewksbury, MA, USA). MS grade water or ultrapure water was purchased from Merck (Darmstadt, Germany) or obtained using a Milli-Q purity system (Millipore, Billerica, MA). Solid phase extraction columns were purchased from Bio-Rad (Hercules, CA, USA).

Synthesis of cis- and trans-3,4-methylene-heptanoylcarnitine

First, both fatty acid isomers were synthesized following a modified protocol as reported in (12, 25) and explained in the **Supplemental figure**. *Cis*-3-heptenoic acid was prepared from *cis*-3-hepten-1-ol using Jones's reagent (CrO_3/H_2SO_4). In a 100 ml three-neck round bottom flask fitted with air condenser, and nitrogen gas flow, *cis*-3-hepten-1-ol (0.4 M) was first dissolved in acetone and placed in an ice bath. Jones's reagent was added dropwise over 10 min and the reaction mixture was stirred for 1 h. The acetone was evaporated under nitrogen gas flow, and the solution was extracted

with diethyl ether. The diethyl ether was extracted with NaOH (3 M), followed by acidification with HCl (5 M) and a second extraction with diethyl ether, dried using anhydrous magnesium sulfate, and evaporated under nitrogen gas flow. The collected *cis*-3-heptenoic acid (53% yield, 98.7% pure) was reacted with diiodomethane following the protocol reported in (22). In a 100 ml three-neck round bottom flask fitted with air condenser and nitrogen gas flow, diethylzinc in hexane (1 M) was added while chilled in an ice-water bath, followed by additions of anhydrous benzene, *cis*-3-heptenoic acid, and diiodomethane. The solution was stirred for three days at room temperature. HCl (1 M) was added for 20 min, and extracted with diethyl ether as described above, and *cis*-3,4-methylene-heptanoic acid (69% yield, 98.0% pure) was collected. For the synthesis of the acylcarnitine ester, *cis*-3,4-methylene-heptanoic acid was reacted with thionyl chloride for 2 h and *cis*-3,4-methylene-heptanoyl chloride was collected. L-carnitine perchlorate was synthesized of L-carnitine hydrochloride and silver perchlorate in anhydrous acetonitrile. The acetonitrile solution was collected, mixed with *cis*-3,4-methylene-heptanoyl chloride, and stirred for 60 h at room temperature. The excess perchlorate was removed with sodium hydrogen carbonate and the product was collected by centrifugation and evaporated to dryness with nitrogen gas (5). The yield was 53%. The *cis*-3,4-methylene-heptanoylcarnitine was purified using solid phase extraction and HPLC and it indicates 98.0% pure by LCMS analyses. The *trans*-isomer was synthesized and purified using the same procedure starting from its respective alcohol, and was 83.0% pure by LCMS analyses.

Analysis of cis- and trans-3,4-methylene-heptanoylcarnitine

UHPLC-Q-TOF/MS/MS using standard solutions of *cis*- and *trans*-3,4-methylene-heptanoylcarnitine (concentrations 20 µg/ml and 15 µg/ml, respectively), a plasma sample (pooled

from all samples from Study 1), and a muscle tissue sample (pooled from all tissue extracts from Study 3) was applied for the identification of *trans*-3,4-methylene-heptanoylcarnitine. The UPLC system (Waters Corp, Milford, USA) was coupled to a Q Exactive HF mass spectrometer (Thermo Fisher, USA). Chromatographic separation was performed with an Acquity BEH C8 column (2.1 mm×100 mm, 1.7 µm). Column temperature was maintained at 50°C. The mobile phase (A) was water containing 0.1% formic acid and (B) was acetonitrile. The analysis started with 5% B kept for 1 min, then B was linearly increased to 100% in 24 min, held for 4 min, then returned back to 5% B, and held finally for 2 min before next injection. The flow rate was 0.35 mL/min. Mass spectrometric detection was performed in positive ion mode. The MS parameters were set as resolution 120,000, capillary temperature 300°C, and spray voltage 3.5 kV. MS/MS fragmentation spectra were generated in the information-dependent acquisition (IDA) auto-MS^2 mode. The IDA-based auto-MS^2 was performed every 0.25 sec, and the 10 most intense metabolite ions in a full scan cycle were used to acquire auto-MS^2. The collision energy voltage was set at 15, 30, and 45 eV.

Quantification of C8:0-carnitine, cis- and trans-3,4-methylene-heptanoylcarnitines in plasma and muscle biopsy samples

Plasma samples were deproteinized with acetonitrile (ratio 1:4) containing isotope labeled d_3-C10:0-carnitine as internal standard (Ten Brink, Amsterdam, Netherlands), run to dryness in a vacuum centrifuge, and reconstituted in acetonitrile/water (4:1). Extraction of freeze-dried muscle biopsy samples was performed by methyl-tert-butyl ether containing d_3-C10:0-carnitine. A mix of the polar and apolar phases was used for analysis. Quantification of C8:0 carnitine, *cis*- and *trans*-3,4-methylene-heptanoylcarnitine in plasma and in muscle tissue was performed by UHPLC-Q-

TOF/MS. The applied analytical conditions were already described in the section above. Integrated peak areas of the samples and the internal standard d_3-C10:0-carnitine were used for quantification. Hepato-splanchnic and leg fluxes (negative=release or positive=uptake) of the acylcarnitines were calculated by multiplying the arterial-to-venous difference by the plasma flow as described in (24).

Statistics

Differences in plasma acylcarnitine concentrations were detected using mixed-model analysis with time as fixed and subject as random effect, followed by Tukey's honestly significant difference (HSD) post-hoc test. Differences in acylcarnitine fluxes over the hepato-splanchnic bed and resting and exercising leg were detected using mixed-model analysis, with resting/exercising leg and time as fixed and subject as random effect, followed by Tukey's HSD test. Statistical analyses and were performed using JMP 14.2.0 (SAS Institute Inc, Cary, North Carolina, USA). A p-value < 0.05 was considered statistically significant. Data are presented as mean ± standard error of the mean (SEM).

RESULTS

C8:1 isomers show different patterns of plasma concentrations compared with other medium-chain acylcarnitines

First, we evaluated the differences in patterns of C8:0 and other medium-chain acylcarnitines compared to C8:1 acylcarnitine isomers in peripheral plasma of exercising humans. In Study 1, comparing plasma levels of medium-chain acylcarnitines obtained at baseline, immediately following 30 min of high intensity exercise corresponding to 105 % of the individual anaerobic lactate threshold, and 120 min into recovery, C8:0 showed a very clear correlation with the other medium-chain acylcarnitines C6:0, C10:0, C10:1, C10-OH, C12:0, and C12:1 (**Fig. 1A**). Two isomers of C8:1 acylcarnitine (C8:1_1, C8:1_2) were detected in the plasma based on m/z and retention time. They showed the best correlation to each other, but less to the other medium-chain acylcarnitines (**Fig. 1A**). C8:0 carnitine plasma concentrations were increased 3-fold and were still higher 120 min after the run (**Fig. 1B**). The concentrations of the C8:1 isomers were marginally elevated by exercise; the magnitude of the increase was >5 times higher for C8:0 than for the two C8:1-species (compare **Fig. 1B, C, D**). The plasma concentrations of the C8:1 isomers differed, e.g., at baseline C8:1_1 (98.2±14.9 nmol/L) vs. C8:1_2 (21.1±3.4 nmol/L), respectively. Altogether, these data pointed to a different metabolism of the two C8:1 isomers compared to C8:0 and other medium-chain acylcarnitines, and raised a question about the structure and origin of these metabolites.

Identification of cis- and trans-3,4-methylene-heptanoylcarnitine in plasma and skeletal muscle biopsy samples

Previously, a slight increase of *cis*-3,4-methylene-heptanoylcarnitine in human plasma during a sub-maximal exercise bout was reported (27), suggesting that the C8:1 metabolites detected in the

current studies could represent this compound, or alternatively could be the *trans*-isomer. To address these questions, *cis*- and *trans*-3,4-methylene-heptanoylcarnitine standards were synthesized and used to elucidate the identity of the C8:1 metabolites. First, we compared the chromatographic and mass spectrometric characteristics of the *cis*- and *trans*-standards of 3,4-methylene-heptanoylcarnitine and performed additionally analyses in biological matrices (pooled plasma and pooled skeletal muscle tissue; see Methods). **Figure 2A** shows in extracted ion chromatograms (EIC) the chromatographic retention of m/z 286.2018, which is the exact mass of 3,4-methylene-heptanoylcarnitine. The retention time (tR) of the *cis*-3,4-methylene-heptanoylcarnitine standard is 6.52 min. It is consistent with the tR of the first peak in plasma and muscle (C8:1_1). The tR of the *trans*-3,4-methylene-heptanoylcarnitine standard (6.64 min) is consistent with the tR of the second peak in the biological samples confirming the identity of the later eluting peak to be the *trans*-isomer (C8:1_2). The minor peak in the EIC of the *trans*-standard may represent an impurity caused by the *cis*-isomer in the standard preparation. In **Figure 2B**, MS/MS fragmentation patterns of the two standards and the two plasma peaks are shown. The parent ion 3,4-methylene-heptanoylcarnitine corresponding to the exact theoretical m/z of 286.2018 is visible in all ESI-MS/MS spectra, which were generated at 15 eV collision energy. In all fragmentation spectra, originating from blood plasma and synthesized *cis*- and *trans*-3,4-methylene-heptanoylcarnitine standards, the fragment ions have identical, characteristic product ions. **Fig. 2C** shows the molecular structure of the characteristic fragmentation of 3,4-methylene-heptanoylcarnitine. The exact mass of the theoretical fragment ions of carnitine are m/z 60.0813, 85.0290, and 144.1025, as well as the neutral loss fragment of m/z 59.0735. The acyl group results in fragments of *m*/z 97.1017 and 125.0966, and trimethylamine leads to a neutral loss fragment ion of *m*/z 227.1283. All these fragment ions were detected, which confirms the identity of 3,4-

methylene-heptanoylcarnitine. Based on the retention time, the minor peak is the *trans*- and the major peak the *cis*-isomer in human plasma and skeletal muscle tissue.

The 3,4-methylene-heptanoylcarnitine isomers are released by the hepato-splanchnic bed and taken up by the exercising skeletal muscle, but only the hepato-splanchnic release of the trans-isomer increases during exercise

Next, we aimed to get deeper insights in the metabolism of the 3,4-methylene-heptanoylcarnitine isomers, particularly on the tissue origins of the metabolites found in plasma. Data of the uptake or release from the hepato-splanchnic bed (Study 2), as well as from the exercising and resting leg and skeletal muscle tissue concentrations (Study 3) were recently published for the *cis*-isomer annotated at that time as "C8:1 carnitine" (24). In Study 2 at baseline, plasma concentration of the *cis*-isomer was 79.8±14.4 nmol/L vs. 11.0±1.5 nmol/l for the *trans*-isomer, and in Study 3 at baseline, plasma concentration of the *cis*-isomer was 139±24.9 nmol/L vs. 22.3±4.7 nmol/L for the *trans*-isomer. Thus, the plasma concentration of the *trans*-isomer was only 10-20 % that of the *cis*-isomer in Study 2 and Study 3, well in line with Study 1 (**Fig. 1C, D**). In contrast, the concentrations of the isomers in skeletal muscle tissue were similar (**Table 1**). In Study 3, both isomers showed an incremental net uptake in the exercising leg, which peaked after 60 min of exercise (**Fig. 3A, B**). This was accompanied by an increase in the skeletal muscle tissue concentration of the exercising leg, but not of the resting leg (**Table 1**). The tissue concentration returned to pre-exercise values 180 min after the exercise bout, likely indicating further metabolism, since we do not detect a net release from the muscle bed at any time point (**Fig. 3A, B**). In contrast to the leg, both isomers showed a clear net release from the hepato-splanchnic bed at all conditions, at baseline, during exercise and in the recovery phase in Study 2 (**Fig. 3C, D**). The flux of the *trans*-isomer over the hepato-splanchnic bed was two-fold higher during recovery after exercise (see 150 and 240 min

compared to baseline values), corresponding to an increase in the flux of approximately 5 nmol/min. The data indicate a clear tissue-specific contribution of the hepato-splanchnic bed and the skeletal muscle to the plasma level of both isomers in resting and exercising conditions.

DISCUSSION

We reported in this work the identification of two C8:1 acylcarnitine isomers, *cis*- and *trans*-3,4-methylene-heptanoylcarnitine, in human plasma and skeletal muscle tissue. The *cis*-isomer of this acylcarnitine with a cyclopropane ring structure was first identified in human urine (25) and later in human plasma (1, 27). The *cis*-3,4-methylene-heptanoylcarnitine was found to moderately increase in plasma (to approximately 1.3-fold of rested state) during an acute 30 min bout of exercise at ~45% of VO_{2max} in obese women before and after a weight loss and fitness intervention (27). This finding was validated in the present study in non-obese trained men, and also found for the *trans*-isomer. These consistent observations across studies are interesting in light of the very different profiles in terms of obesity, sex, and physical fitness across study cohorts. Notably, after the weight-loss and fitness intervention which improved VO_2peak from 21.1 to 25.6 ml/min/kg and reduced fat mass from 41.9 to 36.2 kg, the increase in *cis*-3,4-methylene-heptanoylcarnitine after the 30 min ergometer exercise (performed at the pre-intervention workload) was nearly identical (27). Together, the previous results and the findings herein indicate that during acute exercise the metabolism of xenometabolites is changed, leading to increased production of 3,4-methylene-heptanoylcarnitine derivatives. However, the exercise bout-associated changes in metabolism are not impacted greatly by one's individual fitness or metabolic health status. It is difficult to directly compare concentration changes between the previous reports and the current studies, due to differences in study design, exercise duration and intensity. It will be important in future studies to explore how fluxes and metabolism of these xenometabolites respond to exercise intensity and

fitness status. In contrast, reduced fitness, obesity and metabolic health status may influence the resting plasma levels of *cis*-3,4-methylene-heptanoylcarnitine: after the weight-loss and fitness intervention in the previous study, plasma levels are clearly reduced, but were still much higher compared with the endurance trained and lean subjects in the three study groups of the current work (approx. 500 nmol/L vs. approx. 100 nmol/L) (27).

It has been postulated that the medium-chain acylcarnitines containing a cyclopropane ring structure are carnitine esters formed during the mitochondrial oxidation of the respective fatty acids (25, 27). Bacteria synthesize these fatty acids via cyclopropane fatty acid synthase in their membranes (7) and cyclopropane fatty acids are described in various kind of foods like milk products and bovine meat (16). Antibiotic treatment led to the disappearance of cyclopropane-containing acylcarnitines in urine and they are also not found in urine of newborns (15). Thus, the origin of cyclopropane acylcarnitines could be from oxidation of fatty acids derived from the gut microbiome, which are absorbed and reach the liver via the portal vein, as proposed previously (27). During the hepatic oxidation of these fatty acids, *cis*-3,4-methylene-heptanoylcarnitine is formed and released into the blood stream. The constant release of the *cis*-isomer from the hepatic-splanchnic circulation which is found in the present study provides novel and clear evidence supporting this hypothesis. The origin from the incomplete oxidation of cyclopropane-containing fatty acids is further strengthened by the detection of other cyclopropane ring-containing acylcarnitines in plasma such as *cis*-3,4-methylene-nonanoylcarnitine (27). The detection of the *trans*-3,4-methylene-heptanoylcarnitine in the present study indicates in addition the oxidation of fatty acids with a *trans*-configured cyclopropane ring. Altogether, there is now clear evidence from previous and current results that the 3,4-methylene-heptanoylcarnitine isomers and other cyclopropane ring-containing acylcarnitines are xenometabolite derivatives whose metabolism is significantly and acutely altered by exercise. That we found the hepato-splanchnic bed to be a major

source of plasma 3,4-methylene-heptanoylcarnitine suggests that β-oxidation in the liver underlies exercise-associated differences in plasma levels. These findings do not exclude the possibility that muscle also generates 3,4-methylene-heptanoylcarnitine from incomplete β-oxidation, but this may be minor when compared to the hepato-splanchnic metabolism. Future studies using isotope-labeled metabolites and flux measurements will be needed to fully elaborate the tissue-specific metabolism of these xenometabolites.

The *cis*- and *trans*-isomer of 3,4-methylene-heptanoylcarnitine share great similarities in their tissue fluxes, with a consistently lower concentration of the *trans*-isomer in plasma. In contrast, the tissue concentration of the *cis*- and the *trans*-isomer in skeletal muscle is similar. Analysis of skeletal muscle tissue revealed an approximately 2.5-fold increase of both isomers after the 120 min exercise bout, which is less in relative magnitude when compared to the increase of other medium-chain acylcarnitines in these subjects, as reported recently (14-fold for C6:0, 7.6-fold for C8:0 and 5.9-fold for C10:0 acylcarnitine (24)). It is possible that the increase in skeletal muscle tissue content is due to the huge increase in blood flow in the exercising leg, which resulted in the increased uptake and accumulation of both isomers. Why the absolute increase in muscle can be similar despite a four times lower plasma level of the *trans*-isomer needs further investigation.

A second difference in the regulation of the *cis*- and the *trans*-isomer is the exercise-mediated enhanced release of *trans*-3,4-methylene-heptanoylcarnitine from the hepato-splanchnic bed, which is in contrast to the unaffected constant release of the *cis*-isomer. One explanation is that an increased net release of 5 nmol/min may not reach a significant change of the more than ten times higher net release of the *cis*-isomer. However, a different availability and metabolism of the respective cyclopropane fatty acids during exercise is possible as well. The adipose tissue was very recently discussed as a possible source of some xenolipids and xenometabolites that are released during exercise (6); however, this remains speculative pending studies of tissue-specific flux.

In conclusion, we identified *trans*-3,4-methylene-heptanoylcarnitine as a novel potential xenometabolite derivative in humans, and provide further evidence that xenometabolites contribute to the exercise-induced alterations in the human metabolome in plasma and skeletal muscle tissue. The results also demonstrate that particular care is needed in the interpretation of metabolomics results before isomers are unequivocally identified, and when considering tissue contributors to changes observed in the circulating blood pool. Leveraging metabolomics to explore tissue-specific arterio-to-venous differences holds tremendous promise to uncover new aspects of metabolic physiology linked to exercise, the microbiome, and overall health.

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DISCLOSURE

The authors declare no competing interests.

LEGENDS

Fig.1. C8:1 isomers show different patterns of peripheral plasma concentrations compared with other medium-chain acylcarnitines. (A) Scatterplot matrix illustrating the correlation between the C8-acylcarnitine species C8:0, C8:1_1, and C8:1_2 with C6:0-, C10:0-, C10:1-, C10-OH-, C12:0- and C12:1-carnitine in the time course of an exercise bout performed by young endurance-trained males (Study 1). Concentrations were measured in peripheral plasma of resting subjects, immediately after a 30-min endurance exercise session and following 2h of recovery. n=20*3 time points. Density ellipses set to α=0.95. (B-D) Corresponding levels of the acylcarnitine species (B) C8:0, (C) C8:1_1 and (D) C8:1_2. Concentrations of the acylcarnitine species C8:0, C8:1_1 and C8:1_2 in plasma of resting subjects, immediately after a 30-min endurance exercise session (exercise), and following 2h of recovery. Values are means±SEM of n=20 individuals. Values are means±SEM of n=20 individuals. *$p<0.005$, ** $p<0.0005$ compared to resting state (mixed-model analysis followed by Tukey's post-hoc test); n.s., non significant ($p>0.05$).

Fig.2. Identification of *cis*- and *trans*-3,4-methylene-heptanoylcarnitine in plasma and skeletal muscle biopsy samples. (A) Extracted ion chromatograms (EIC) of the mass of 3,4-methylene-heptanoylcarnitine. EICs of blood plasma, skeletal muscle tissue extract and synthesized *cis*- and *trans*-3,4-methylene-heptanoylcarnitine are given. (B) ESI-MS/MS fragmentation spectra of blood plasma and synthesized *cis*- and *trans*-3,4-methylene-heptanoylcarnitine at 15eV collision energy. The first peak corresponds to C8:1_1, the second peak to C8:1_2. (C) Illustration of molecular

structures of characteristic fragment ions of 3,4-methylene-heptanoylcarnitine shown in the fragmentation pattern of the *trans*-isomer generated from the blood plasma sample.

Fig.3. Comparison of the net fluxes of *cis*- and *trans*-3,4-methylene heptanoylcarnitine over the resting and exercising legs (Study 3; A,B) as well as the hepato-splanchnic bed (Study 2; C,D) in young male subjects. Grey areas indicate baseline and time during exercise 0-120 min). # $p<0.05$ compared to time point 0, * $p<0.05$ compared to resting leg according to mixed-model analysis and TukeyHSD post-hoc test. Values are means ±SEM of n=8 (liver) or n=9 (leg study) individuals; one-leg: only n=8 for 60 min and 150 min due to missing samples. Values of the *cis*-isomer were already published in (24), as “C8:1”.

Table 1

Tissue concentration of *cis*- and *trans*-3,4-methylene-heptanoylcarnitine in skeletal muscle of exercising humans (Study 3)

	0 min		120 min (acute exercise)		300 min (recovery)	
3,4-methylene-heptanoylcarnitine (nmol/mg tissue)	Resting leg	Exercise leg	Resting leg	Exercise leg	Resting leg	Exercise leg
cis-isomer[1)]	1.41±0.38	1.29±0.25	1.20±0.25	3.18±0.62	1.56±0.36	1.85±0.34
trans-isomer	1.31±0.31	1.13±0.20	1.05±0.18	3.11±0.62	1.38±0.33	1.56±0.23

[1)]Values of the *cis*-isomer were already published in (24), as “C8:1”.

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Figure 1

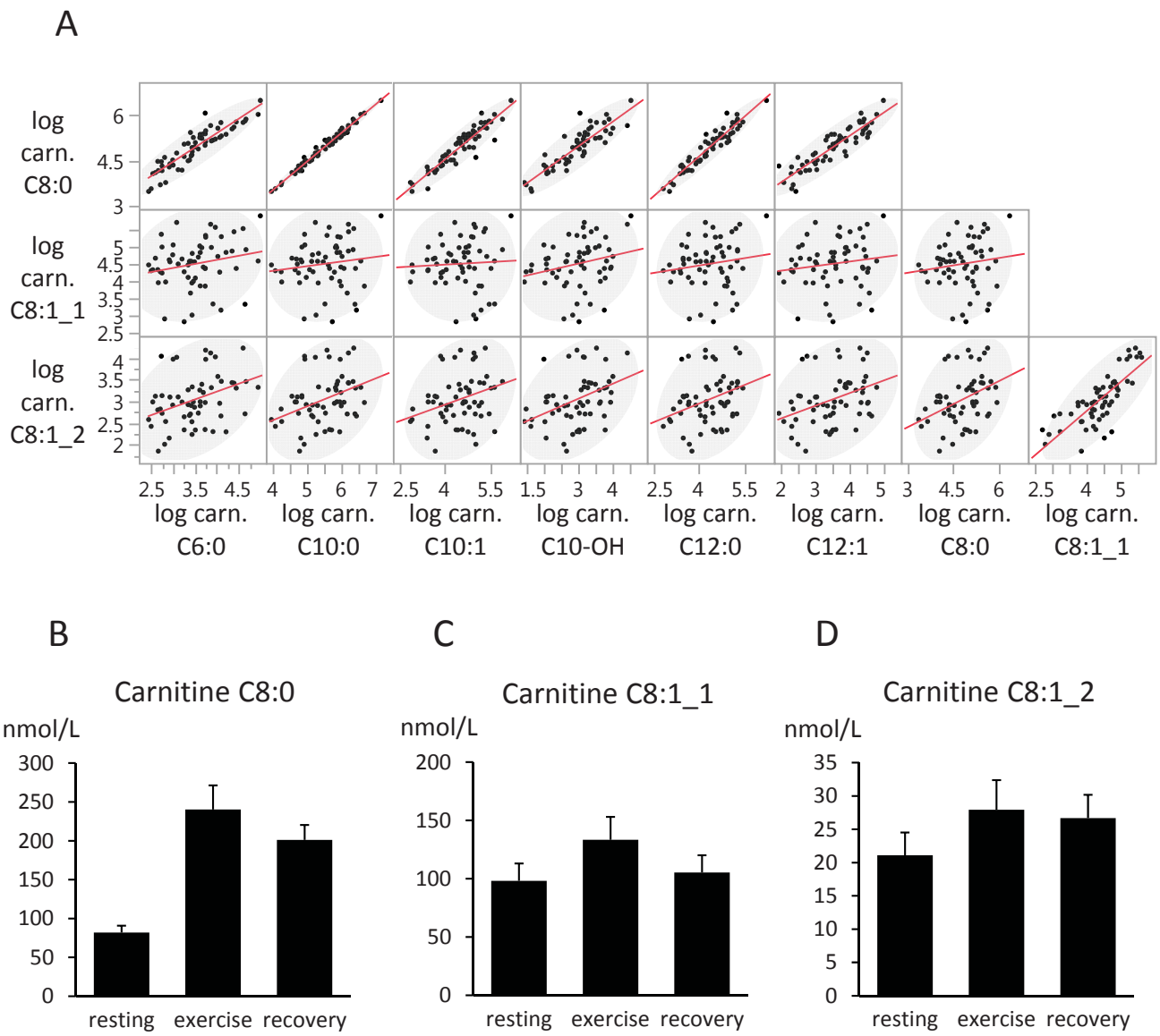
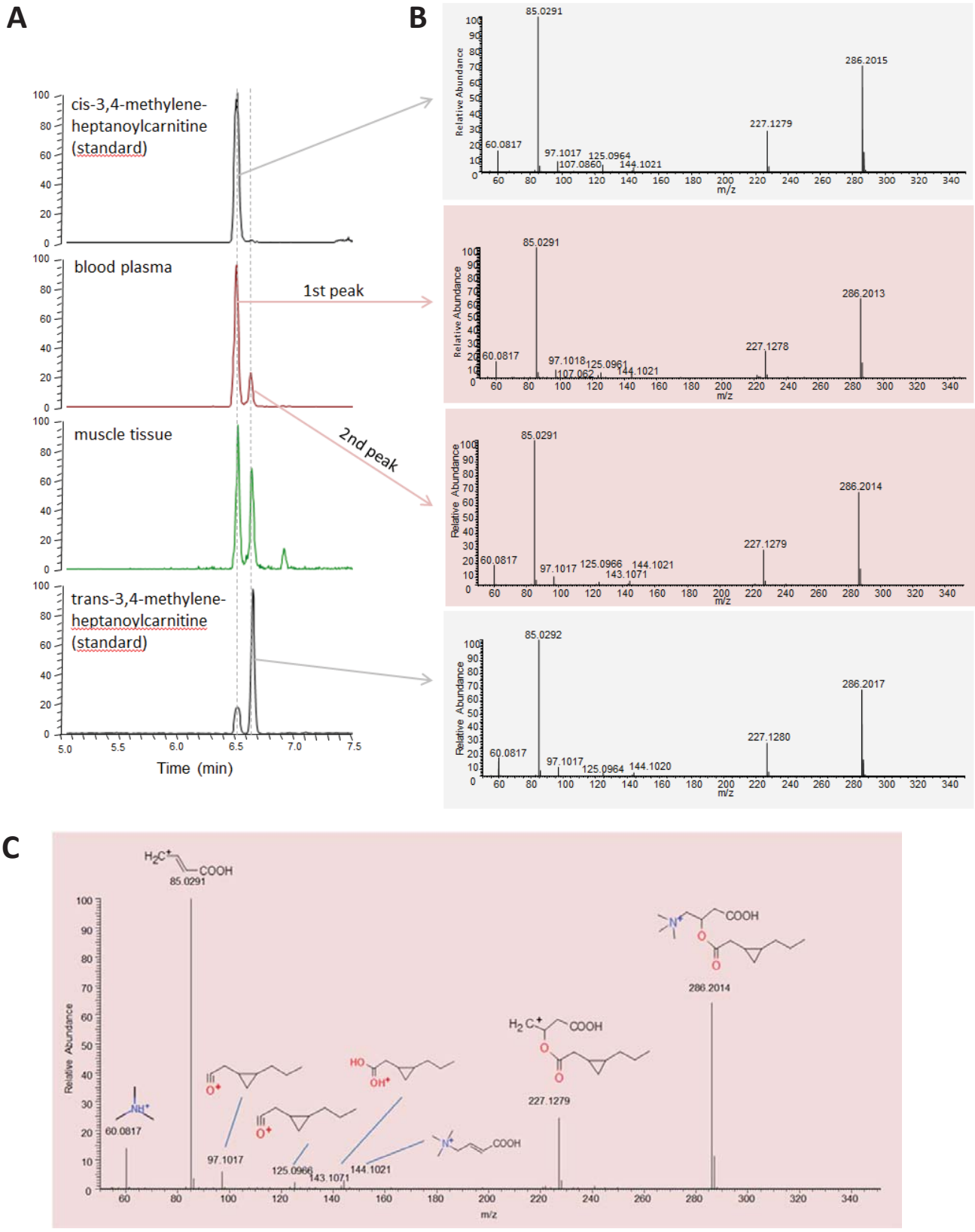

A
log carn. C8:0
log carn. C8:1_1
log carn. C8:1_2
log carn. C6:0
log carn. C10:0
log carn. C10:1
log carn. C10-OH
log carn. C12:0
log carn. C12:1
log carn. C8:0
log carn. C8:1_1
B
Carnitine C8:0
nmol/L
resting
exercise
recovery
C
Carnitine C8:1_1
nmol/L
resting
exercise
recovery
D
Carnitine C8:1_2
nmol/L
resting
exercise
recovery

Figure 2


A
B
C
cis-3,4-methylene-heptanoylcarnitine (standard)
blood plasma
1st peak
muscle tissue
2nd peak
trans-3,4-methylene-heptanoylcarnitine (standard)
Time (min)
Relative Abundance
m/z
85.0291
286.2015
227.1279
60.0817
97.1017
125.0964
107.0860
144.1021
85.0291
286.2013
227.1278
60.0817
97.1018
125.0961
107.062
144.1021
85.0291
286.2014
227.1279
60.0817
97.1017
125.0966
144.1021
143.1071
85.0292
286.2017
227.1280
60.0817
97.1017
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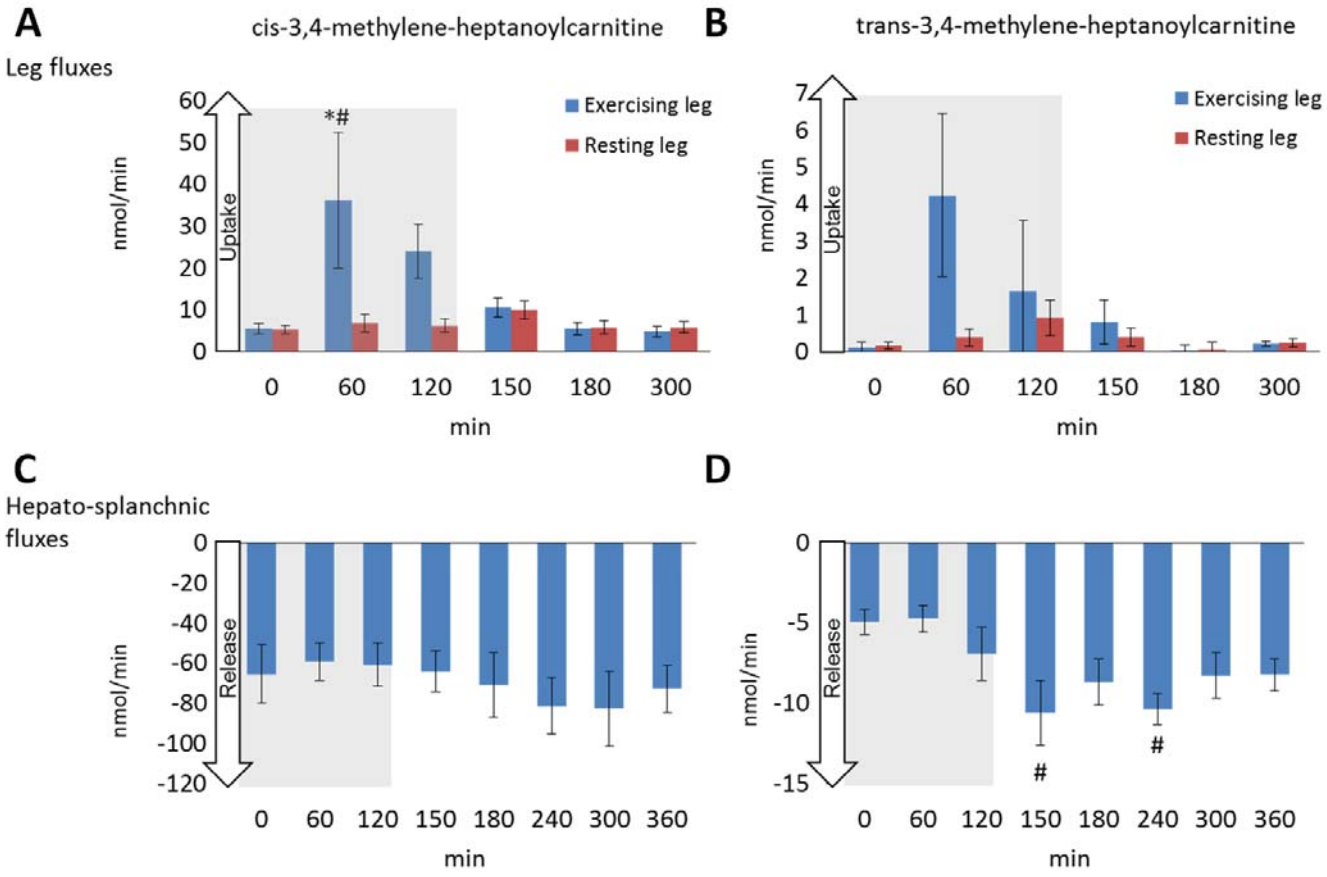
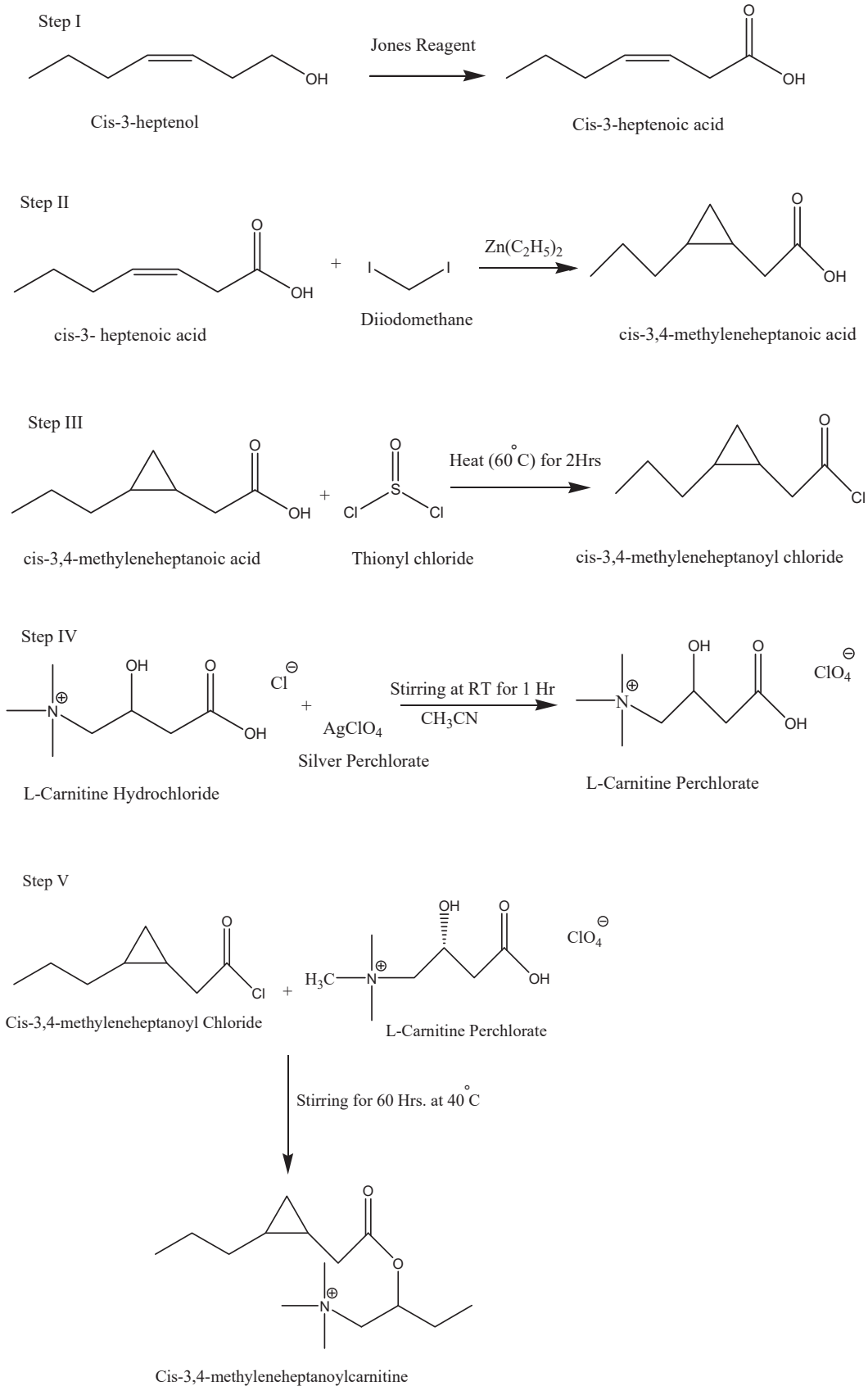
97.1017
125.0966
143.1071
144.1021

Figure 3


A
Leg fluxes
cis-3,4-methylene-heptanoylcarnitine
B
trans-3,4-methylene-heptanoylcarnitine
Exercising leg
Resting leg
nmol/min
Uptake
*#
min
C
Hepato-splanchnic fluxes
D
Release
#

Supplemental Figure



Scheme of the synthesis of cis-3,4-methylene-heptanoylcarnitine as described in Methods.