Am J Physiol Endocrinol Metab 318: E701–E709, 2020.
First published February 26, 2020; doi:10.1152/ajpendo.00510.2019.

RESEARCH ARTICLE

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

Hany F. Sobhi,[1]* Xinjie Zhao,[2]* Peter Plomgaard,[3,4,5] Miriam Hoene,[6] Jakob S. Hansen,[3,4] Benedikt Karus,[7] Andreas M. Niess,[7] Hans U. Häring,[8,9] Rainer Lehmann,[6,8,9] Sean H. Adams,[10,11] Guowang Xu,[2] and Cora Weigert[6,8,9]

[1]*Department of Natural Sciences, Center for Organic Synthesis, Coppin State University, Baltimore, Maryland;* [2]*CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Dalian, China;* [3]*Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark;* [4]*The Centre of Inflammation and Metabolism and the Centre for Physical Activity Research, Department of Infectious Diseases and CMRC, Rigshospitalet, Copenhagen, Denmark;* [5]*Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark;* [6]*Institute for Clinical Chemistry and Pathobiochemistry, University Hospital, Tuebingen, Germany;* [7]*Department for Sports Medicine, University Hospital, Tuebingen, Germany;* [8]*Institute for Diabetes Research and Metabolic Diseases, Helmholtz Zentrum Muenchen, University of Tuebingen, Tuebingen, Germany;* [9]*German Center for Diabetes Research, Oberschleissheim, Germany;* [10]*Arkansas Children's Nutrition Center, Little Rock, Arkansas; and* [11]*Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas*

Submitted 19 November 2019; accepted in final form 24 February 2020

Sobhi HF, Zhao X, Plomgaard P, Hoene M, Hansen JS, Karus B, Niess AM, Häring HU, Lehmann R, Adams SH, Xu G, Weigert C. Identification and regulation of the xenometabolite derivatives *cis*- and *trans*-3,4-methylene-heptanoylcarnitine in plasma and skeletal muscle of exercising humans. *Am J Physiol Endocrinol Metab* 318: E701–E709, 2020. First published February 26, 2020; doi:10.1152/ajpendo.00510.2019.—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 *cis*-isomer, in plasma and skeletal muscle 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 hepatosplanchnic bed and the exercising leg in plasma samples obtained in three separate studies from young, lean and healthy males. Compared with other medium-chain acylcarnitines, the plasma concentrations of the 3,4-methylene-heptanoylcarnitine isomers only marginally increased with exercise. Both isomers showed a more than twofold increase in the skeletal muscle tissue of the exercising leg; this may have been due to the net effect of fatty acid oxidation in the exercising muscle and uptake from blood. The latter idea is supported by a more than twofold increased net uptake in the exercising leg only. Both isomers showed a constant release from the hepatosplanchnic 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 regardless of the exercise state. This is the first approach studying kinetics and fluxes of xenolipid isomers from tissues under exercise 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 xenometabolites, opening perspectives for future studies about the physiological role of this largely unknown class of metabolites.

arterial-to-venous difference; exercise; liver; microbiome; xenolipids

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 acylcarnitine 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 our knowledge of the complexity of acylcarnitine species by comprehensive large-scale acylcarnitine identification resulting in a database of 758 acylcarnitines, comprising 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 and 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)physio-

* H. F. Sobhi and X. Zhao contributed equally to this study.

Correspondence: C. Weigert (e-mail: cora.weigert@med.uni-tuebingen.de); G. Xu (e-mail: xugw@dicp.ac.cn).

logical relevance. Along these lines, a pronounced increase in plasma medium-chain acylcarnitines is a significant feature in exercising humans (10, 14, 27). We (24) recently reported a medium-chain acylcarnitine species showing an unusual time course, i.e., almost no increase in plasma during and after 2 h cycling exercise at 60% maximal O_2 uptake. 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 acyl group contains a cyclopropane ring, which has been described only 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 that 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 hepatosplanchnic 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 hepatosplanchnic 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 Ethics 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°C until ultrahigh-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 yr; 20.8 ± 0.2 kg/m^2 BMI; 66.2 ± 1.7 mL·kg^{-1}·min^{-1} $\dot{V}O_{2max}$) who had a light breakfast (2 pretzels of ~200 kcal per pretzel) and water 90 min before baseline blood sampling.

In *study 2*, 10 healthy male subjects (age: 22.9 ± 0.8 yr; BMI: 22.6 ± 0.5 kg/m^2) performed a 120 min-cycling exercise at 60% $\dot{V}O_{2max}$ in semisupine position after an overnight fast; subjects remained fasting throughout the whole experimental period (9). Briefly, catheters were inserted into the brachial artery of the nondominant 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 with the other, resting leg (8). Briefly, after an overnight fast, nine healthy male subjects (age: 20.9 ± 0.5 yr; 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 contralateral leg was resting. Catheters were inserted retrograde into both femoral veins as well as into one femoral artery. The subjects were fasted until 3 h after the exercise bout. Blood flow was determined for both the resting and the exercising leg by 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, and 300 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). *Cis*-3-hepten-1-ol and *trans*-3-hepten-1-ol were purchased from Alfa Aesar (Tewksbury, MA). MS-grade water or ultrapure water was purchased from Merck or obtained using a Milli-Q purity system (Millipore, Billerica, MA). Solid-phase extraction columns were purchased from Bio-Rad (Hercules, CA).

Synthesis of cis- and trans-3,4-methylene-heptanoylcarnitine. First, both fatty acid isomers were synthesized following a modified protocol as reported (12, 25) and explained in Supplemental Fig. S1 (Supplemental material is available at https://doi.org/10.6084/m9.figshare.11808507.v1). *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 a reported protocol (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 3 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% purity by LC-MS analyses. The *trans*-isomer was synthesized and purified using the same procedure, starting from its respective alcohol and was 83.0% pure by LC-MS 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, MA) was coupled to a Q Exactive HF mass spectrometer (Thermo Fisher). 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, and then B was linearly increased to 100% in 24 min, held for 4 min, and then returned back to 5% B and held finally for 2 min before next injection. The flow rate was 0.35 mL/min. MS 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 s, 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 medium-chain acylcarnitines 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, The 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 nonpolar phases was used for analysis. Quantification of medium-chain acylcarnitines in plasma and in muscle tissue was performed by UHPLC-Q-TOF/MS. Samples from *studies 1–3* were analyzed at the same laboratory site. The applied analytic 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. Hepatosplanchnic and leg fluxes (negative = release, positive = uptake) of the acylcarnitines were calculated by multiplying the arterial-to-venous difference by the plasma flow, as described in Supplemental methods (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 hepatosplanchnic 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 were performed using JMP 14.2.0 (SAS Institute, Inc., Cary, NC). A P value of <0.05 was considered statistically significant. Data are presented as means ± SE.

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 with 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. 1*A*). 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. 1*A*). C8:0 carnitine plasma concentrations were increased threefold and were still higher 120 min after the run (Fig. 1*B*). The concentrations of the C8:1 isomers were marginally elevated by exercise; the magnitude of the increase was more than five times higher for C8:0 than for the two C8:1-species (compare Fig. 1, *B*, *C*, and *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). All together, these data pointed to a different metabolism of the two C8:1 isomers compared with 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 submaximal 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 additional analyses in biological matrices (pooled plasma and pooled skeletal muscle tissue; see METHODS). Figure 2*A* 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-

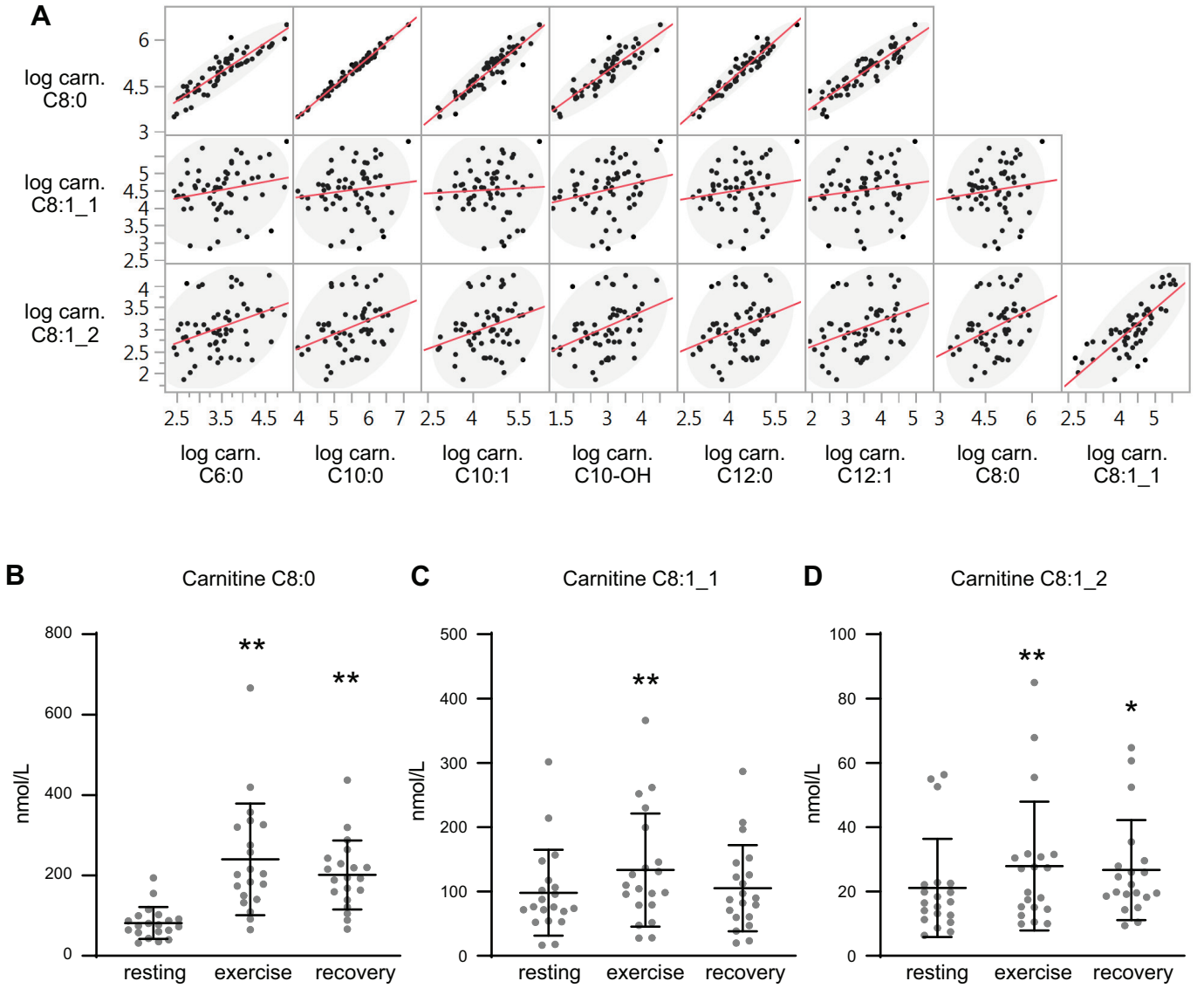

Fig. 1. C8:1 isomers show different patterns of peripheral plasma concentrations compared with other medium-chain acylcarnitines. *A*: scatterplot matrix illustrating the correlation between the C8-acylcarnitine species C8:0, C8:1_1, and C8:1_2 with C6:0-, C10:0-, C10:1-, C10-OH-, C12:0-, and C12:1-carnitine 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 2 h of recovery; $n = 20$ at 3 time points. Density ellipses set to $\alpha = 0.95$. *B–D*: corresponding levels of acylcarnitine species C8:0 (*B*), C8:1_1 (*C*), and C8:1_2 (*D*) in plasma of resting subjects immediately after a 30-min endurance exercise session (exercise), and following 2 h of recovery. Values are shown as scatter dot plot with lines indicating means ± SD; $n = 20$ individuals. $^{*}P < 0.005$, $^{**}P < 0.0005$ vs. resting state (mixed-model analysis followed by Tukey's post hoc test).

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 Fig. 2*B*, 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. Figure 2*C* 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.

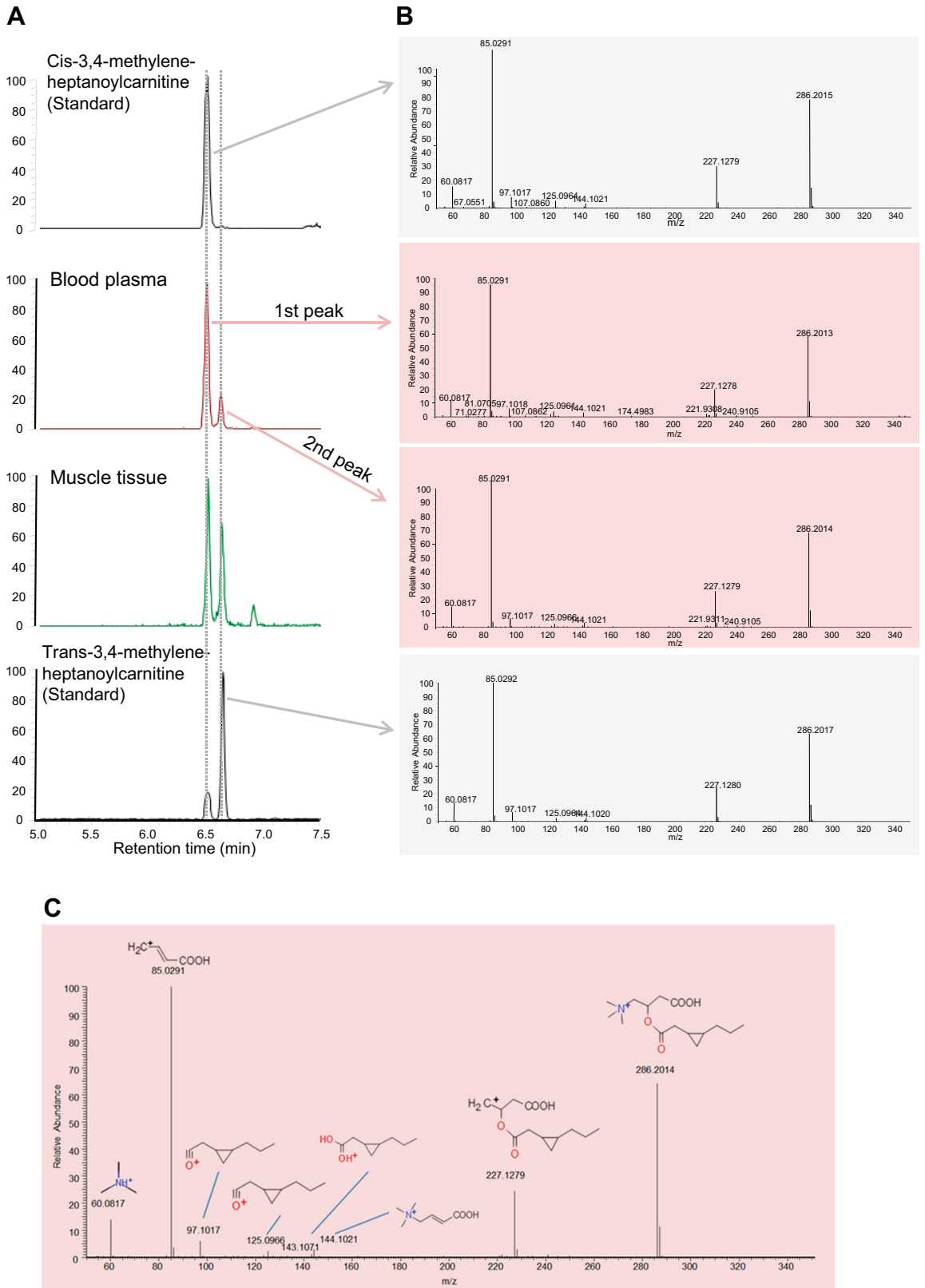

Fig. 2. Identification of *cis*- and *trans*-3,4-methylene-heptanoylcarnitine in plasma and skeletal muscle biopsy samples. *A*: extracted ion chromatograms (EIC) of the mass of 3,4-methylene-heptanoylcarnitine. EICs of blood plasma, skeletal muscle tissue extract, and synthesized *cis*- and *trans*-3,4-methylene-heptanoylcarnitine are given. *B*: ESI-MS/MS fragmentation spectra of blood plasma and synthesized *cis*- and *trans*-3,4-methylene-heptanoylcarnitine at 15 eV 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.

The 3,4-methylene-heptanoylcarnitine isomers are released by the hepatosplanchnic bed and taken up by the exercising skeletal muscle, but only the hepatosplanchnic 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 hepatosplanchnic 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. 1, *C* and *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. 3, *A* and *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 preexercise values 180 min after the exercise bout, likely indicating further metabolism, since we did not detect a net release from the muscle bed at any time point (Fig. 3, *A* and *B*). In contrast to the leg, both isomers showed a clear net release from the hepatosplanchnic bed at all conditions, at baseline, during exercise, and in the recovery phase in *study 2* (Fig. 3, *C* and *D*). The flux of the *trans*-isomer over the hepatosplanchnic bed was twofold higher during recovery after exercise (see 150 and 240 min vs. baseline values), corresponding to an increase in the flux of ~5 nmol/min. The data indicate a clear tissue-specific contribution of the hepatosplanchnic bed and the skeletal muscle to the plasma level of both isomers in resting and exercising conditions.

DISCUSSION

We have 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 ~1.3-fold of resting state) during an acute 30-min bout of exercise at ~45% of $\dot{V}O_{2max}$ in obese women before and after a weight loss and fitness intervention (27). This finding was validated in the present study in nonobese 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 $\dot{V}O_{2peak}$ from 21.1 to 25.6 $mL \cdot min^{-1} \cdot kg^{-1}$ 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 preintervention 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 and exercise duration and intensity. It will be important in future studies to explore how the 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 were clearly reduced but were still much higher compared with the endurance-trained and lean subjects in the three study groups of the current work (~500 nmol/L vs. ~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 "parent" long-chain 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 kinds 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 microbiota, which are absorbed and reach the liver via the portal vein and/or released from adipose storage, as proposed previously (27). Under this model, during the hepatic and tissue oxidation of these fatty acids, *cis*-3,4-methylene-heptanoylcarnitine is formed and released into the bloodstream. The constant release of the *cis*-isomer from the hepatosplanchnic circulation that 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 the oxidation of fatty acids with a *trans*-configured

Table 1. *Tissue concentration of cis- and trans-3,4-methylene-heptanoylcarnitine in skeletal muscle of exercising humans (study 3)*

3,4-Methylene-heptanoylcarnitine, nmol/mg tissue	0 min		120 min (Acute Exercise)		300 min (Recovery)	
	Resting leg	Exercise leg	Resting leg	Exercise leg	Resting leg	Exercise leg
Cis-isomer	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

Values are means ± SE. Values of the *cis*-isomer were already published (24), as "C8:1". $^{**}P < 0.0001$ vs. exercise leg at 0 min; $^{\#\#}P < 0.0001$ vs. resting leg at 120 min according to mixed-model analysis and Tukey HSD post hoc test.

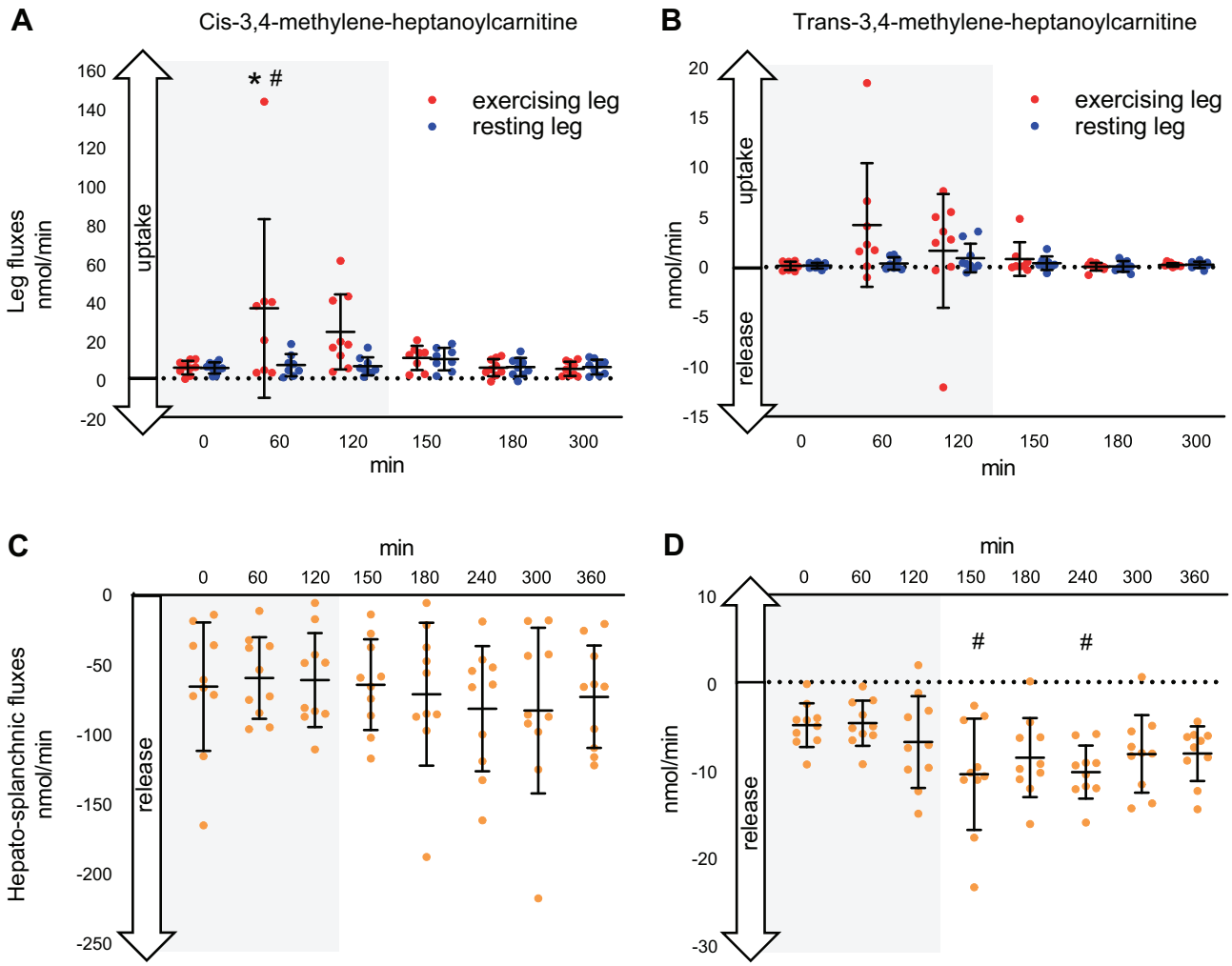

Fig. 3. Comparison of net fluxes of *cis*- and *trans*-3,4-methylene heptanoylcarnitine over resting and exercising legs (*study 3*; *A* and *B*) as well as the hepatosplanchnic bed (*study 2*; *C* and *D*) in young male subjects. Gray areas indicate baseline and time during exercise 0–120 min. #$P < 0.05$ vs. time point 0; *$P < 0.05$ vs. resting leg according to mixed-model analysis and Tukey HSD post hoc test. Values are shown as scatter dot plot with lines indicating means ± SD; $n = 8$ (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 (24), as "C8:1".

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 hepatosplanchnic bed to be a major source of plasma 3,4-methylene-heptanoylcarnitine suggests that β-oxidation in the liver underlies most of the exercise-associated differences in plasma levels. These findings do not exclude the possibility that muscle or other tissues also generate 3,4-methylene-heptanoylcarnitine from incomplete β-oxidation, but this may be minor compared with the hepatosplanchnic metabolism. Future studies using isotope-labeled metabolites and flux measurements will be needed to fully elaborate the tissue-specific metabolism of these xenometabolites. That the gut microbiota are the potential origin of the cyclopropane ring-containing fatty acids opens the possibility to influence the plasma and tissue concentrations of 3,4-methylene-heptanoylcarnitine by dietary intervention and probiotics.

The *cis*- and *trans*-isomers 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 concentrations of the *cis*- and the *trans*-isomers in skeletal muscle are similar. Analysis of skeletal muscle tissue revealed an ~2.5-fold increase of both isomers after the 120-min exercise bout, which is less in relative magnitude than 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 was due to the increase in blood flow in the exercising leg, which resulted in the increased uptake and accumulation of both isomers. Why the absolute increases 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*-isomers is the exercise-mediated enhanced release of *trans*-3,4-methylene-heptanoylcarnitine from the hepatosplanchnic 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. 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.

The subjects in the three study groups of the present paper were not identical, and the exercise trials and blood sampling were conducted at three different laboratory sites, which might be considered a limitation. However, the subjects in the three studies were of similar age, BMI, and high physical fitness, which is reflected by the comparable plasma levels of the 3,4-methylene-heptanoylcarnitine isomers. It must also be acknowledged that our studies were designed to investigate the splanchnic origin and exercise responsiveness of the xenolipids, but they cannot inform on potential biological activities of the cyclopropane ring-containing fatty acids and their acylcarnitine derivatives. Careful studies addressing specific hypotheses need to be conducted to shed light on the biological importance of this class of lipids in terms of potential bioactivities, metabolism, and tissue trafficking.

In conclusion, we identified *trans*-3,4-methylene-heptanoylcarnitine as a novel potential xenometabolite derivative in humans, and have provided 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 tissue contributions to changes observed in the circulating blood pool are considered. Leveraging metabolomics to explore tissue-specific arterial-to-venous differences holds tremendous promise to uncover new aspects of metabolic physiology linked to exercise, the microbiome, and overall health.

GRANTS

This work was supported, in part, by United States Department of Agriculture-Agricultural Research Service (USDA-ARS) Projects 6026-51000-010-05S and 6026-51000-012-06S (to S.H.A. and H.F.S.). This study was further supported by a grant from the Danish Diabetes Academy supported by the Novo Nordisk Foundation to C.W., by the Augustinus Foundation, and in part by grants from the German Federal Ministry of Education and Research (BMBF) to the German Centre for Diabetes Research (DZD e.V.; No. 01GI0925), by grants of the Sino-German Center for Research Promotion (GZ 753 by DFG and NSFC to G.X. and R.L., and L.E., 1391/1-1 by DFG to R.L.); by the key foundations (21934006) from the National Natural Science Foundation of China to G.X.. The Centre for Physical Activity Research (CFAS) is supported by a grant from TrygFonden. The Copenhagen Muscle Research Centre (CMRC) is supported by a grant from the Capital Region of Denmark. During the study period, the Centre of Inflammation and Metabolism (CIM) was supported by a grant from the Danish National Research Foundation (DNRF55). CIM/CFAS is a member of DD2, the Danish Center for Strategic Research in Type 2 Diabetes (the Danish Council for Strategic Research, grant nos. 09-067009 and 09-075724).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

H.F.S., P.P., J.S.H., A.M.N., H.U.H., R.L., S.H.A., G.X., and C.W. conceived and designed research; H.F.S., X.Z., P.P., J.S.H., and B.K. performed experiments; H.F.S., X.Z., P.P., M.H., A.M.N., and C.W. analyzed data; P.P., A.M.N., H.U.H., R.L., S.H.A., and C.W. interpreted results of experiments; X.Z. and M.H. prepared figures; P.P. and M.H. drafted manuscript; R.L., S.H.A., G.X., and C.W. edited and revised manuscript; H.F.S., X.Z., P.P., M.H., J.S.H., B.K., A.M.N., H.U.H., R.L., S.H.A., G.X., and C.W. approved final version of manuscript.

REFERENCES

1. **Adams SH, Hoppel CL, Lok KH, Zhao L, Wong SW, Minkler PE, Hwang DH, Newman JW, Garvey WT.** Plasma acylcarnitine profiles suggest incomplete long-chain fatty acid beta-oxidation and altered tricarboxylic acid cycle activity in type 2 diabetic African-American women. *J Nutr* 139: 1073–1081, 2009. doi:10.3945/jn.108.103754.
2. **Anderson SG, Dunn WB, Banerjee M, Brown M, Broadhurst DI, Goodacre R, Cooper GJ, Kell DB, Cruickshank JK.** Evidence that multiple defects in lipid regulation occur before hyperglycemia during the prodrome of type-2 diabetes. *PLoS One* 9: e103217, 2014. doi:10.1371/journal.pone.0103217.
3. **Baker PR II, Boyle KE, Koves TR, Ilkayeva OR, Muoio DM, Houmard JA, Friedman JE.** Metabolomic analysis reveals altered skeletal muscle amino acid and fatty acid handling in obese humans. *Obesity (Silver Spring)* 23: 981–988, 2015. doi:10.1002/oby.21046.
4. **Bao X, Katz S, Pollard M, Ohlrogge J.** Carbocyclic fatty acids in plants: biochemical and molecular genetic characterization of cyclopropane fatty acid synthesis of Sterculiafoetida. *Proc Natl Acad Sci USA* 99: 7172–7177, 2002. doi:10.1073/pnas.092152999.
5. **Brendel K, Bressler R.** The resolution of (plus or minus)-carnitine and the synthesis of acylcarnitines. *Biochim Biophys Acta* 137: 98–106, 1967. doi:10.1016/0005-2760(67)90012-4.
6. **Grapov D, Fiehn O, Campbell C, Chandler CJ, Burnett DJ, Souza EC, Casazza GA, Keim NL, Newman JW, Hunter GR, Fernandez JR, Garvey WT, Hoppel CL, Harper ME, Adams SH.** Exercise plasma metabolomics and xenometabolomics in obese, sedentary, insulin-resistant women: impact of a fitness and weight loss intervention. *Am J Physiol Endocrinol Metab* 317: E999–E1014, 2019. doi:10.1152/ajpendo.00091.2019.
7. **Grogan DW, Cronan JE Jr.** Cyclopropane ring formation in membrane lipids of bacteria. *Microbiol Mol Biol Rev* 61: 429–441, 1997. doi:10.1128/.61.4.429-441.1997.
8. **Hansen J, Brandt C, Nielsen AR, Hojman P, Whitham M, Febbraio MA, Pedersen BK, Plomgaard P.** Exercise induces a marked increase in plasma follistatin: evidence that follistatin is a contraction-induced hepatokine. *Endocrinology* 152: 164–171, 2011. doi:10.1210/en.2010-0868.
9. **Hansen JS, Clemmesen JO, Secher NH, Hoene M, Drescher A, Weigert C, Pedersen BK, Plomgaard P.** Glucagon-to-insulin ratio is pivotal for splanchnic regulation of FGF-21 in humans. *Mol Metab* 4: 551–560, 2015. doi:10.1016/j.molmet.2015.06.001.
10. **Hansen JS, Zhao X, Irmler M, Liu X, Hoene M, Scheler M, Li Y, Beckers J, Hrabě de Angelis M, Häring HU, Pedersen BK, Lehmann R, Xu G, Plomgaard P, Weigert C.** Type 2 diabetes alters metabolic and transcriptional signatures of glucose and amino acid metabolism during exercise and recovery. *Diabetologia* 58: 1845–1854, 2015. doi:10.1007/s00125-015-3584-x.
11. **Hiatt WR, Regensteiner JG, Wolfel EE, Ruff L, Brass EP.** Carnitine and acylcarnitine metabolism during exercise in humans. Dependence on skeletal muscle metabolic state. *J Clin Invest* 84: 1167–1173, 1989. doi:10.1172/JCI114281.
12. **Jin SJ, Tserng KY.** Metabolic origins of urinary unsaturated dicarboxylic acids. *Biochemistry* 29: 8540–8547, 1990. doi:10.1021/bi00489a006.
13. **Kalim S, Clish CB, Wenger J, Elmariah S, Yeh RW, Deferio JJ, Pierce K, Deik A, Gerszten RE, Thadhani R, Rhee EP.** A plasma long-chain acylcarnitine predicts cardiovascular mortality in incident dialysis patients. *J Am Heart Assoc* 2: e000542, 2013. doi:10.1161/JAHA.113.000542.
14. **Lehmann R, Zhao X, Weigert C, Simon P, Fehrenbach E, Fritsche J, Machann J, Schick F, Wang J, Hoene M, Schleicher ED, Häring HU, Xu G, Niess AM.** Medium chain acylcarnitines dominate the metabolite pattern in humans under moderate intensity exercise and support lipid oxidation. *PLoS One* 5: e11519, 2010. doi:10.1371/journal.pone.0011519.
15. **Libert R, Van Hoof F, Laus G, De Nayer P, Habib Jiwan JL, de Hoffmann E, Schanck A.** Identification of ethylsuccinylcarnitine present in some human urines. *Clin Chim Acta* 355: 145–151, 2005. doi:10.1016/j.cccn.2004.12.020.
16. **Lolli V, Dall'Asta M, Del Rio D, Palla G, Caligiani A.** Presence of cyclopropane fatty acids in foods and estimation of dietary intake in the Italian population. *Int J Food Sci Nutr* 70: 467–473, 2019. doi:10.1080/09637486.2018.1540556.

17. **McCoin CS, Knotts TA, Adams SH.** Acylcarnitines—old actors auditioning for new roles in metabolic physiology. *Nat Rev Endocrinol* 11: 617–625, 2015. doi:10.1038/nrendo.2015.129.
18. **Mihalik SJ, Goodpaster BH, Kelley DE, Chace DH, Vockley J, Toledo FG, DeLany JP.** Increased levels of plasma acylcarnitines in obesity and type 2 diabetes and identification of a marker of glucolipotoxicity. *Obesity (Silver Spring)* 18: 1695–1700, 2010. doi:10.1038/oby.2009.510.
19. **Mihalik SJ, Michaliszyn SF, de las Heras J, Bacha F, Lee S, Chace DH, DeJesus VR, Vockley J, Arslanian SA.** Metabolomic profiling of fatty acid and amino acid metabolism in youth with obesity and type 2 diabetes: evidence for enhanced mitochondrial oxidation. *Diabetes Care* 35: 605–611, 2012. doi:10.2337/DC11-1577.
20. **Ramos-Roman MA, Sweetman L, Valdez MJ, Parks EJ.** Postprandial changes in plasma acylcarnitine concentrations as markers of fatty acid flux in overweight and obesity. *Metabolism* 61: 202–212, 2012. doi:10.1016/j.metabol.2011.06.008.
21. **Soeters MR, Sauerwein HP, Duran M, Wanders RJ, Ackermans MT, Fliers E, Houten SM, Serlie MJ.** Muscle acylcarnitines during short-term fasting in lean healthy men. *Clin Sci (Lond)* 116: 585–592, 2009. doi:10.1042/CS20080433.
22. **Tanaka A, Nishizaki T.** The newly synthesized linoleic acid derivative FR236924 induces a long-lasting facilitation of hippocampal neurotransmission by targeting nicotinic acetylcholine receptors. *Bioorg Med Chem Lett* 13: 1037–1040, 2003. doi:10.1016/S0960-894X(03)00089-1.
23. **Van Hove JL, Zhang W, Kahler SG, Roe CR, Chen YT, Terada N, Chace DH, Iafolla AK, Ding JH, Millington DS.** Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency: diagnosis by acylcarnitine analysis in blood. *Am J Hum Genet* 52: 958–966, 1993.
24. **Xu G, Hansen JS, Zhao XJ, Chen S, Hoene M, Wang XL, Clemmesen JO, Secher NH, Häring HU, Pedersen BK, Lehmann R, Weigert C, Plomgaard P.** Liver and muscle contribute differently to the plasma acylcarnitine pool during fasting and exercise in humans. *J Clin Endocrinol Metab* 101: 5044–5052, 2016. doi:10.1210/jc.2016-1859.
25. **Yang S, Minkler P, Hoppel C.** cis-3,4-Methylene-heptanoylcarnitine: characterization and verification of the C8:1 acylcarnitine in human urine. *J Chromatogr B Analyt Technol Biomed Life Sci* 857: 251–258, 2007. doi:10.1016/j.jchromb.2007.07.027.
26. **Yu D, Zhou L, Xuan Q, Wang L, Zhao X, Lu X, Xu G.** Strategy for comprehensive identification of acylcarnitines based on liquid chromatography-high-resolution mass spectrometry. *Anal Chem* 90: 5712–5718, 2018. doi:10.1021/acs.analchem.7b05471.
27. **Zhang J, Light AR, Hoppel CL, Campbell C, Chandler CJ, Burnett DJ, Souza EC, Casazza GA, Hughen RW, Keim NL, Newman JW, Hunter GR, Fernandez JR, Garvey WT, Harper ME, Fiehn O, Adams SH.** Acylcarnitines as markers of exercise-associated fuel partitioning, xenometabolism, and potential signals to muscle afferent neurons. *Exp Physiol* 102: 48–69, 2017. doi:10.1113/EP086019.