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ORIGINAL ARTICLE

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Carnitine supplementation improves insulin sensitivity and skeletal muscle acetylcarnitine formation in patients with type 2 diabetes

Yvonne M. H. Op den Kamp-Bruls PhD[1] | Yvo J. M. Op den Kamp MD[2] | Pandichelvam Veeraiah PhD[1] | Ruben Zapata Perez PhD[3] | Esther Phielix PhD[2] | Bas Havekes MD[4] | Gert Schaart PhD[2] | Esther Kornips[2] | Bart R. B. Berendsen[5] | Ashraf Virmani PhD[5] | Joachim E. Wildberger MD[1] | Riekelt H. Houtkooper PhD[3] | Matthijs K. C. Hesselink PhD[2] | Patrick Schrauwen PhD[6,7] | Vera B. Schrauwen-Hinderling PhD[1,2,7,8]

[1]Departments of Radiology and Nuclear Medicine, NUTRIM School for Nutrition and Translational Research in Metabolism, Maastricht University Medical Center, Maastricht, The Netherlands

[2]Department of Nutrition and Movement Sciences, NUTRIM School for Nutrition and Translational Research in Metabolism, Maastricht University Medical Center, Maastricht, The Netherlands

[3]Laboratory Genetic Metabolic Diseases, Amsterdam Gastroenterology and Metabolism, Amsterdam Cardiovascular Sciences, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, Netherlands

[4]Department of Internal Medicine, Division of Endocrinology, NUTRIM School for Nutrition and Translational Research in Metabolism, Maastricht University Medical Center, Maastricht, The Netherlands

[5]Alfasigma Nederland B.V., Utrecht, The Netherlands

[6]Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands

[7]Institute for Clinical Diabetology, German Diabetes Center, Leibniz Institute for Diabetes Research at Heinrich Heine University Düsseldorf, Düsseldorf, Germany

[8]German Center for Diabetes Research (DZD), Partner Düsseldorf, Neuherberg, Germany

Correspondence
Vera B. Schrauwen-Hinderling, Department of Radiology and Nuclear Medicine, Department of Nutrition and Movement Sciences, Maastricht University Medical Center, 6200 MD Maastricht, The Netherlands.
Email: v.schrauwen@maastrichtuniversity.nl

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Abstract

Aim/Hypothesis: Recently, we reported that increasing free carnitine availability resulted in elevated skeletal muscle acetylcarnitine concentrations and restored metabolic flexibility in individuals who have impaired glucose tolerance. Metabolic flexibility is defined as the capacity to switch from predominantly fat oxidation while fasted to carbohydrate oxidation while insulin stimulated. Here we investigated if carnitine supplementation enhances the capacity of skeletal muscle to form acetylcarnitine and thereby improves insulin sensitivity and glucose homeostasis in patients with type 2 diabetes (T2DM).

Methods: Thirty-two patients followed a 12-week L-carnitine treatment (2970 mg/day, orally). Insulin sensitivity was assessed by a two-step hyperinsulinemic-euglycemic clamp. In vivo skeletal muscle acetylcarnitine concentrations at rest and post-exercise (30 min, 70% W_{max}) and intrahepatic lipid content (IHL) were

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determined by proton magnetic resonance spectroscopy (^{1}H-MRS). All measurements were performed before and after 12 weeks of carnitine supplementation.

Results: Compliance with the carnitine supplementation was good (as indicated by increased plasma-free carnitine levels ($p < 0.01$) and pill count (97.1 ± 0.7%)). Insulin-induced suppression of endogenous glucose production (31.9 ± 2.9 vs. 39.9 ± 3.2%, $p = 0.020$) and peripheral insulin sensitivity (Δ rate of glucose disappearance (ΔR_d): 10.53 ± 1.85 vs. 13.83 ± 2.02 μmol/kg/min, $p = 0.005$) improved after supplementation. Resting (1.18 ± 0.13 vs. 1.54 ± 0.17 mmol/kgww, $p = 0.008$) and post-exercise (3.70 ± 0.22 vs. 4.53 ± 0.30 mmol/kgww, $p < 0.001$) skeletal muscle acetylcarnitine concentrations were both elevated after carnitine supplementation. Plasma glucose ($p = 0.083$) and IHL ($p = 0.098$) tended to be reduced after carnitine supplementation.

Conclusion: Carnitine supplementation improved insulin sensitivity and tended to lower IHL and fasting plasma glucose levels in patients with type 2 diabetes. Furthermore, carnitine supplementation increased acetylcarnitine concentration in muscle, which may underlie the beneficial effect on insulin sensitivity.

KEYWORDS

acetylcarnitine, carnitine supplementation, hyperinsulinemic-euglycemic clamp, insulin sensitivity, intrahepatic lipid content, magnetic resonance spectroscopy, type 2 diabetes

1 | INTRODUCTION

The number of people diagnosed with type 2 diabetes mellitus (T2DM) is increasing rapidly worldwide, reaching pandemic proportions.[1] In the aetiology of T2DM, the development of insulin resistance is an important hallmark, driving hyperglycaemia,[2] but the underlying mechanisms leading to insulin resistance are not fully elucidated.

Acetylcarnitine gained attention as an important metabolite in light of maintaining insulin sensitivity and glucose homeostasis.[3–6] In animal models, decreased skeletal muscle acetylcarnitine concentrations are reported in obese insulin-resistant mice, along with blunted metabolic flexibility and elevated plasma glucose levels,[5] where metabolic flexibility is defined as the ability to switch from predominantly fat oxidation while fasted to carbohydrate oxidation upon insulin stimulation.[2] We previously developed a novel non-invasive proton magnetic resonance spectroscopy (^{1}H-MRS) method to enable the determination of in vivo skeletal muscle acetylcarnitine concentrations in humans.[3] Applying this technique, we showed that insulin sensitivity correlates positively with in vivo magnetic resonance (MR)-based skeletal muscle acetylcarnitine concentration,[3] indicating lower acetylcarnitine levels in the muscle of insulin-resistant individuals and patients with type 2 diabetes.

Acetylcarnitine is formed upon transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to free carnitine, which is mediated via the enzyme carnitine acetyltransferase (CrAT).[4,6,7] A positive correlation was found between free carnitine availability and acetylcarnitine in muscle tissue,[6] suggesting the importance of freely available carnitine for the formation of acetylcarnitine. Carnitine is best known for its role in translocating long-chain acyl-CoAs from the cytosol into the mitochondria to facilitate fat oxidation.[6,7] In addition, carnitine is also involved in removing redundant acyl chains from the mitochondria by forming acylcarnitines, including acetylcarnitine.[4–7] When carbon load via acyl chains is high in the mitochondria, such as during (over) feeding and exercise, substrate supply exceeds substrate utilization, and carnitine can facilitate the conversion of excessive intramitochondrial acyl-CoA intermediates towards acylcarnitines, including acetylcarnitine, which in turn can be exported out of the mitochondria.[7,8] This export mechanism for excessive substrate is suggested to be very important in preserving metabolic flexibility and thereby metabolic health.[4–7] Animal studies indeed revealed a link between reduced free carnitine availability and insulin resistance and metabolic inflexibility.[5,6,8]

We recently showed that carnitine supplementation in prediabetic individuals elevated MR-based skeletal muscle acetylcarnitine levels and improved metabolic flexibility.[9] However, insulin sensitivity remained unchanged in these individuals, which could be attributed to the short supplementation period of 4 weeks.[10] In patients with T2DM, positive results of oral carnitine supplementation have been reported on fasting plasma glucose (FPG),[11–14] fasting insulin,[12,14] $HbA1_c$[14] and markers of insulin sensitivity as assessed by Homeostatic Model Assessment for Insulin Resistance (HOMA-IR).[10–12,14] These results point towards improved insulin sensitivity by carnitine in T2DM, but this has never been investigated using the gold standard 2-step hyperinsulinemic-euglycemic clamp procedure, which evaluates both hepatic and peripheral insulin sensitivity. Furthermore, the

mechanism underlying improved insulin sensitivity in humans remains elusive, and it is unclear whether an increased formation of acetylcarnitine may explain such improvements. We here hypothesize that the positive effects of carnitine on insulin sensitivity and glucose homeostasis in T2DM depend on the improvement of the capacity to form acetylcarnitine. Therefore, we here investigated if carnitine supplementation for 3 months elevates skeletal muscle acetylcarnitine while also improving insulin sensitivity and glucose homeostasis in patients with T2DM. We also investigated whether the acetylcarnitine concentrations prior to the start of the study are predictive of the response to the supplementation. As an explorative objective, we also investigated whether (potential) improvements in insulin sensitivity by carnitine translate into benefits in daily life functioning (i.e. physical and cognitive performance) since previous research indicated beneficial effects on these parameters after carnitine supplementation.[15]

2 | METHODS

2.1 | Ethical approval

The medical-ethical review committee of the University Hospital Maastricht and Maastricht University approved all study procedures in accordance with the declaration of Helsinki. Trial monitoring was performed by the Clinical Trial Center Maastricht. The study was registered at clinicaltrials.gov with identifier NCT03230812; the first registration date is July 26, 2017. Written informed consent was given by all participants.

2.2 | Participants

Thirty-two patients with overweight/obesity (BMI: 25–38 kg/m^2), who were middle-aged (40–75 years) and have T2DM, were included in this study (Figure 1). Both male and post-menopausal female patients with T2DM who had stable dietary habits and were on oral glucose-lowering medication but did not use other medication interfering with the investigated study measurements (as determined by the responsible physician) were included. Patients with T2DM were either on diet ($n = 5$), metformin ($n = 15$), sulfonylurea ($n = 3$) or metformin + sulfonylurea ($n = 9$) therapy for at least 6 months prior to the start of the study. At screening, criteria were assessed and evaluated by a medical doctor. Exclusion criteria were the use of insulin therapy, uncontrolled diabetes (HbA1c > 9.5%), clinically relevant diabetes-related complications (i.e. active diabetic foot, polyneuropathy or retinopathy), uncontrolled hypertension (>140/100 mmHg), medical history or active cardiovascular disease (Electrocardiogram, ECG), impaired kidney and/or renal function (among other based on plasma ASAT, ALAT, GGT, creatinine and bilirubin levels), anaemia (Hb <12.5 g/L), MRI contra-indications, unstable body weight (weight gain or loss >5 kg in the previous 3 months), engagement in more than 3 h of exercise a week, and being vegetarian (because of altered whole body carnitine status).

2.3 | Experimental design

The study was set up as an intervention trial performed between March 2018 and November 2019. All patients underwent baseline measurements (visits 1, 2, 3 and 4, Figure 2) followed by 3 months (96 days) of oral carnitine supplementation (next to the participants' usual medication). Between days 90 and 96 of the carnitine supplementation period, measurements were repeated (visits 7, 8 and 9, Figure 2). Compliance was checked during a monthly visit by counting unused supplements and furthermore by measuring plasma-free carnitine levels at baseline and after 3 months. Participants were asked to maintain their usual physical activity patterns and regular diet during participation in the study. Changes in physical activity levels were monitored by Baecke's habitual physical activity questionnaire at baseline and 3 months.[16] To prevent additional intake of oral carnitine, consumption of food supplements was not allowed. Participants refrained from alcohol and strenuous physical activity 3 days prior to a test day.

2.4 | Treatment

All participants received oral supplementation with carnitine chewing tablets (2970 mg/day of L-Carnitine (Alfasigma Nederland BV, Utrecht, The Netherlands)) for 3 months (equal to 96 days). The carnitine dosage was based on previous studies in humans reporting improvements in insulin sensitivity (HOMA-IR) and glucose tolerance after 3000 mg/day of oral carnitine supplementation.[13,17] Participants consumed nine chewing tablets (à 330 mg) per day: three tablets in the morning during breakfast, three tablets during lunch and three tablets during dinner. On the morning of the hyperinsulinemic-euglycemic clamp (day 96), participants did not take any carnitine.

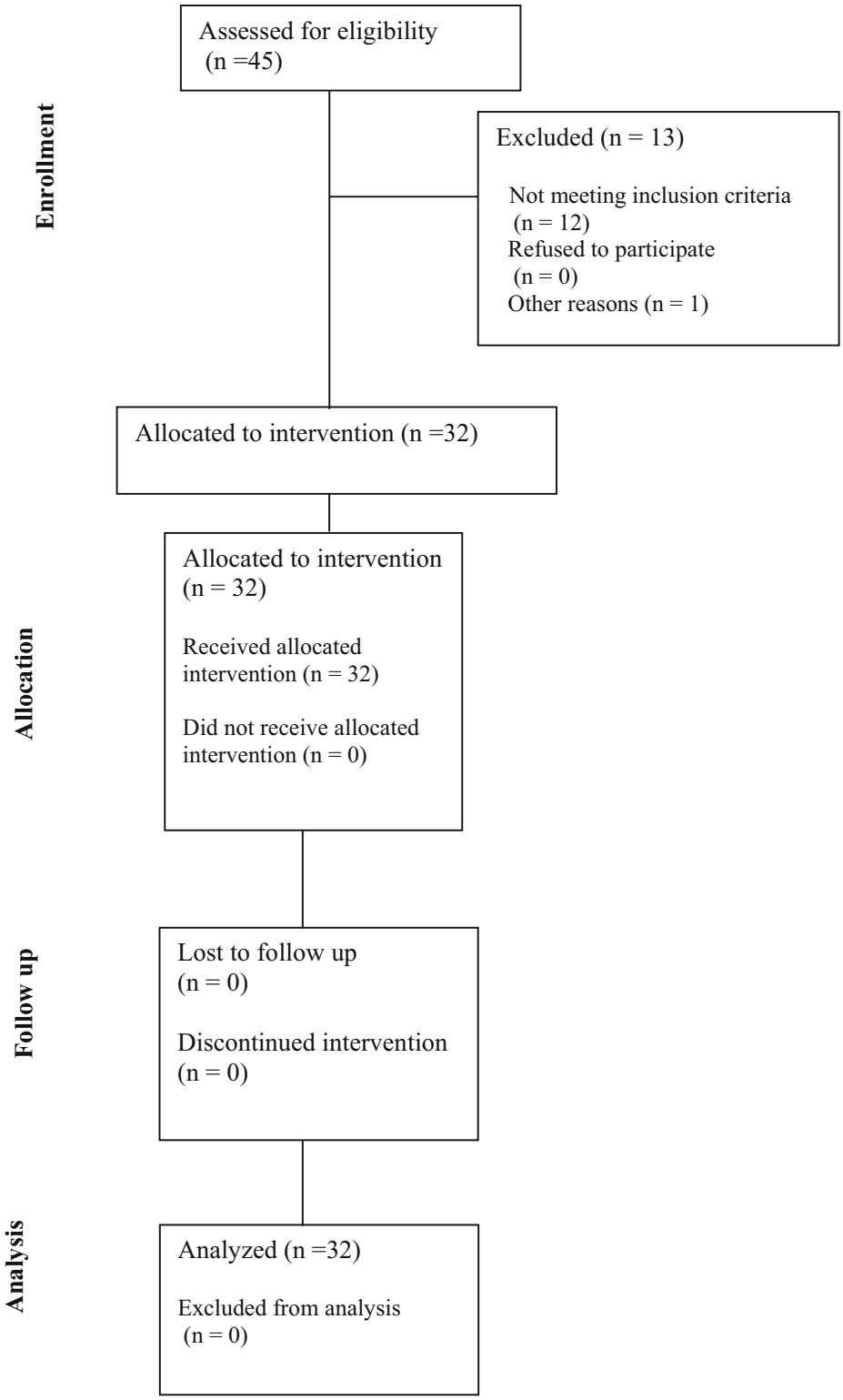
2.5 | Outcomes

The primary study endpoint was peripheral insulin sensitivity (expressed as ΔR_d), as measured by a hyperinsulinemic-euglycemic clamp. Other endpoints were acetylcarnitine concentrations before and after exercise, metabolic flexibility, intrahepatic lipid content, body composition, plasma metabolites (i.e. glucose, HbA1c and acylcarnitines), and patients perceived benefits in general daily functioning (i.e. physical and cognitive performance, quality of life and quality of sleep).

2.6 | VO_{2max}

For characterization (visit 1), participants performed a graded cycling test on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) to determine maximal oxygen uptake (VO_{2max}) and maximal power output (W_{max}). O_2 consumption and CO_2 production were measured using an Oxycon beta, and RER was

FIGURE 1 Consort diagram.



monitored as a verification of the subjects' maximal effort. After a warming-up period of 5 min, at a start workload of 75 watts, the workload was increased by 50 watts every 2.5 min. When the heart rate of subjects reached 80% of their predicted maximal heart rate (220-age), the workload was increased by 25 watts until exhaustion was reached or until the subject was no longer able to keep their speed of rotation above 60 revolutions per minute.[18]

2.7 | ^{1}H-MRS acetylcarnitine

At visit 2 and 7 (day 90), participants consumed a light lunch at noon (12:00 PM) and remained fasted for the following 5 h. Participants refrained from physical activity and reported to the laboratory at 4:30 PM. After arrival, subjects rested for 30 min, and at 5:00 PM, resting skeletal muscle acetylcarnitine concentrations were assessed

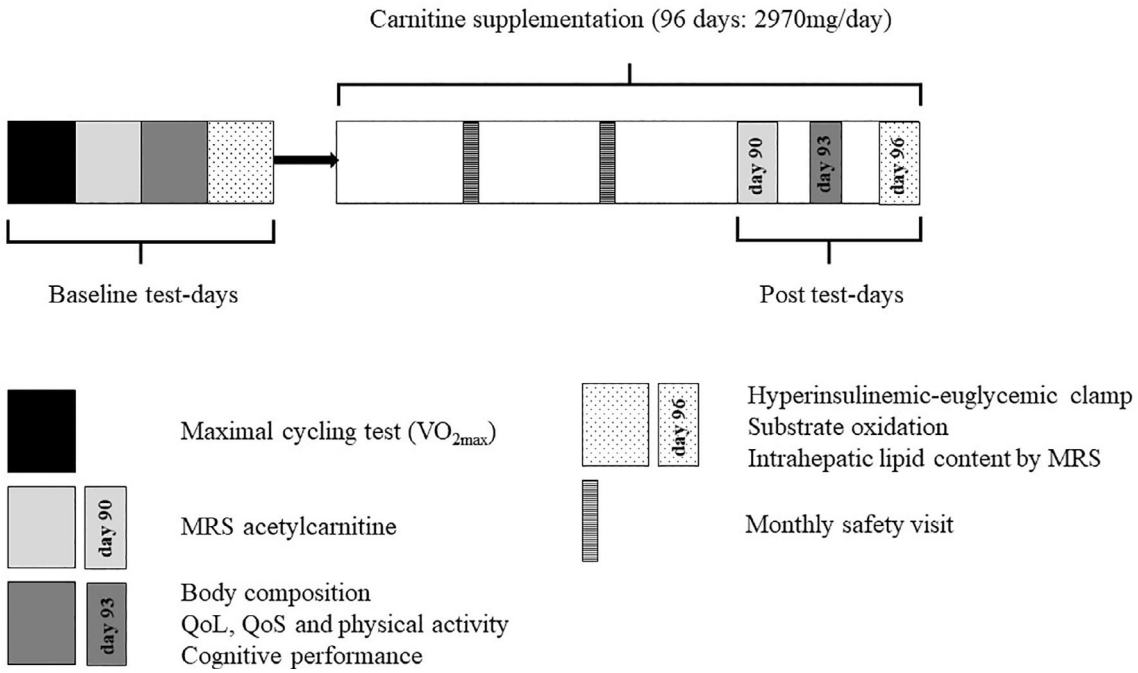


FIGURE 2 Study outline of the 96-day carnitine supplementation interventional trial in type 2 diabetes patients. Participants performed four baseline test days followed by 3 months (equal to 96 days) of oral carnitine supplementation. Post-test days were conducted during the last week of supplementation (days 90, 93 and 96). MRS, magnetic resonance spectroscopy; QoL, quality of life; QoS, quality of sleep. For one participant, the supplementation period was prolonged due to personal circumstances (99 days), all other participants adhered to the 96-day schedule.

by ^{1}H-MRS. Subsequently, as high-intensity exercise is known to stimulate the formation of acetylcarnitine,[19,20] participants performed a 30-minute cycling exercise at 70% of the participants' predetermined maximal power output (W_{max}). Directly after cycling, skeletal muscle acetylcarnitine concentrations were quantified again.

Skeletal muscle acetylcarnitine concentrations were determined in vivo as previously validated and reported on a 3 T clinical MR scanner (Achieva 3 T-X, Philips Healthcare, Best, The Netherlands).[21] Spectra were analysed with a custom-made MATLAB script (The Mathworks Inc., Natrick, USA) and acetylcarnitine concentrations were calculated as previously described.[3]

2.8 | Body composition

During visits 3 and 8, body mass and body volume were determined with a Bod Pod device (Cosmed, Rome, Italy) using air-displacement plethysmography (ADP) and fat percentage was calculated as described previously.[22] The measurement was performed in the morning (08.00 AM) after an overnight fast.

2.9 | Physical performance

In the late morning (10.30 AM) of visits 3 and 8, two physical performance tests were conducted: a Timed Chair-Stand Test (TCST) and a 6-Minute Walk Test (6MWT). During the TCST, participants performed 10 chair-rise repetitions as fast as possible, while performance time was recorded. For the 6MWT, participants were instructed to walk (no running) as far as possible within 6 min on a 10 m flat track.

2.10 | Quality of sleep and quality of life

The Pittsburgh Sleep Quality Index (PSQI) was used to estimate quality of sleep (QoS) over the previous month.[23] The PSQI consisted of a 10-item questionnaire resulting in a score between 0 and 21, with a lower score indicating a better sleep quality. Quality of life (QoL) was assessed by a 32-item survey.[24] Domains of social, emotional, cognitive, physical and spiritual well-being were included and contributed to a combined QoL score. The survey ranges between 32 and 160 points, with a higher score indicating a better QoL.

2.11 | Cognitive performance

Cognitive performance was assessed at visits 3 and 8 using the Cambridge neuropsychological test automated battery (CANTAB). All participants performed the CANTAB battery in a quiet chamber in the fasting state. Three different cognitive domains were investigated: psychomotor speed (selecting specific information), executive function (planning, decision-making and impulse control) and memory (ability to store and retrieve information). Psychomotor speed was

determined using the motor screening task (MOT) and reaction time (RT). Executive function was assessed via multitasking tests (MTT) and spatial span (SSP), while delayed matching to sample (DMS) and paired associates learning (PAL) were used to investigate memory. Data analysis of MOT, RT, MTT, SSP, DMS and PAL was performed as previously described.[25]

2.12 | Hyperinsulinemic-Euglycemic two-step clamp

At visits 4 and 9, all participants came to the university (06:00 AM) after an overnight fast and underwent a two-step hyperinsulinemic-euglycemic clamp with co-infusion of D-[6,6-$^2H_2$]-glucose tracer (16.8 mg/mL, 0.04 mL/kg/min) to determine insulin sensitivity as previously described.[26] After starting the glucose tracer infusion, a muscle biopsy was taken from the *m. vastus lateralis* under local anaesthesia (1% lidocaine, without epinephrine) using the Bergström technique.[27]

After 180 min of glucose tracer infusion (sufficient time to reach an equilibrium in patients with type 2 diabetes), a low-dose insulin infusion (10 mU/m^2/min) was started for 3 h to assess hepatic insulin sensitivity, followed by 2.5 h of high dose insulin infusion (40 mU/m^2/min) to measure peripheral insulin sensitivity. During the steady states ($t = 150–180$ min, $t = 330–360$ min, and $t = 480–510$ min) blood samples were collected every 10 min (4 times per steady state) and indirect calorimetry was performed for 30 min using a ventilated hood (Omnical, IDEE, Maastricht, The Netherlands) to determine VO2 and VCO2. A fixed flow was applied of ~90 L/min. Carbohydrate and fat oxidation rates were calculated using the formulas of Frayn et al.,[28] and protein oxidation was assumed to be 15% energy expenditure. The respiratory exchange ratio (RER), defined as VCO_2/VO_2, was used to determine metabolic flexibility (ΔRER). Metabolic flexibility (ΔRER) reflects the difference between the insulin-stimulated RER and RER at basal conditions. Isotopic enrichment of plasma glucose was quantified by electron ionization gas chromatography–mass spectrometry as previously described.[29] Steele's single pool non-steady state equations were used (to correct for potential small deviations from steady states) to calculate glucose appearance (R_a) and disappearance (R_d).[30] Non-oxidative glucose disposal (NOGD) was calculated as R_d minus carbohydrate oxidation (determined with indirect calorimetry) Volume of distribution was assumed to be 0.190 L/kg for glucose.

2.13 | ^{1}H-MRS intrahepatic lipid

At visit 4 and 9, proton magnetic resonance spectroscopy (^{1}H-MRS) was performed prior to the start of the insulin infusion of the hyperinsulinemic-euglycemic clamp (07:00 AM) to determine intrahepatic lipid content (IHL). IHL content was determined on a 3 T clinical MR scanner (Achieva 3 T-X, Philips Healthcare, Best, The Netherlands). A STEAM sequence was used to acquire spectra using the following acquisition parameters: repetition time (TR) = 4500 ms, echo time (TE) = 20 ms, mixing time (TM) = 16 ms, spectral bandwidth = 2 kHz, number of acquired data points = 2048, volume of interest (VOI) = 30×30×30 mm, and the number of signal averages (NSA) = 128. VAPOUR water suppression was applied, and an additional water reference scan (NSA = 16) was obtained. Spectra were post-processed in a home-written MATLAB (MATLAB 2014b, The MathWorks, Inc., Natick, Massachusetts, United States) script. Target (lipid-CH_2 and water) resonances were fitted individually in the respective spectrum. IHL was expressed in absolute (weight/weight) percentage using the ratio of T_2 corrected signal intensities of lipid-CH_2 and water.

2.14 | Plasma and muscle metabolites

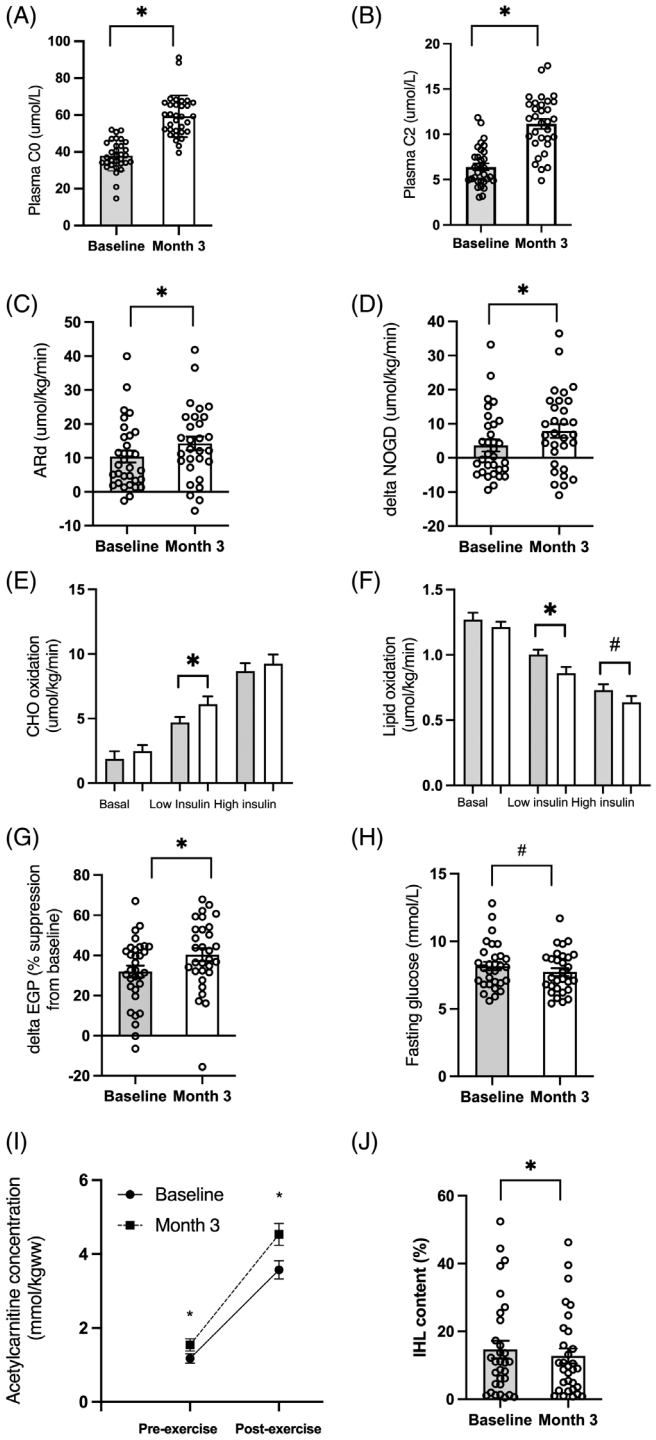
Plasma metabolites were analysed at baseline and after 3 months of carnitine supplementation. Acylcarnitine species were analysed via tandem mass spectrometry as previously described.[31] Glucose (Horiba, Montpellier, France), free fatty acids (Wako Chemicals, Neuss, Germany), triglycerides (Sigma, St. Louis, USA) and cholesterol (Roche Diagnostics, Mannheim, Germany) concentrations were determined colorimetrically on the Cobas Pentra analyzer (Horiba, Montpellier, France). HbA_{1c} levels were determined using the NU kit (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands), and plasma and insulin levels were determined via a human insulin Elisa kit (Crystal Chem, Elk Grove Village, USA). Skeletal muscle metabolomics was performed as previously described.[32] In brief, freeze-dried muscle tissue (2–4 mg) was transferred to 2-mL tubes, and then 425 µL water, 500 µL methanol, and 75 µL of internal standards mixture (see Table S1) were added to each sample. Samples were homogenized using TissueLyser II (Qiagen; 5 min at 30/s), followed by the addition of 1000 µL chloroform and thorough mixing. After centrifugation, the top layer containing the polar phase was transferred to 1.5-mL tubes and dried in a vacuum evaporator at 60°C. Dried samples were reconstituted in 100 µL methanol/water (6/4, v/v) and analysed in an Aquity UPLC system (Waters) coupled to an Impact IITM Ultra-High Resolution qTOF mass spectrometer (Bruker). Chromatographic separation of the compounds was achieved using a SeQuant ZIC-cHILIC column (PEEK 100 × 2.1 mm, 3 µm particle size, Merck, Darmstadt, Germany) at 30°C. The LC method consisted of a gradient running at 0.25 mL/min from 100% mobile phase B (9:1 acetonitrile:water with 5 mM ammonium acetate pH 6.8) to 100% mobile phase A (1:9 acetonitrile:water with 5 mM ammonium acetate pH 6.8) in 28 min, followed by a re-equilibration step at 100% B for 5 min. MS data were acquired both in negative and positive ionization modes in full scan mode over the range of m/z 50–1200.

Metabolite identification has been based on a combination of accurate mass, (relative) retention times, and fragmentation spectra compared with the analysis of a library of standards (Sigma-Aldrich MSMLS).

2.15 | Sample size calculation and statistics

The sample size was chosen in order to be able to detect a clinically significant improvement in insulin sensitivity of 8%–15% as assessed

during a hyperinsulinemic-euglycemic clamp.[33,34] Taking the intraindividual variation of the difference in insulin sensitivity in repeated measurements of 7%–15% into account,[35,36] the chosen sample size of $N = 32$ was amply sufficient (for $\beta > 80\%$, $\alpha < 0.05$, two-sided testing). Data are presented as means ± SEM and p-values of <0.05 were considered significantly different (two-sided testing, $\alpha = 0.05$, $1-\beta$ of 80%). Statistical analyses were conducted using SPSS 24.0 software (IBM Corp: IBM SPSS statistics for Windows, Armonk New York, USA). Normal distribution of the data was evaluated using a Shapiro–Wilk normality test. Student paired sample t-tests were performed to investigate differences before and after 3 months of oral carnitine intake if data were normally distributed. In case data were not normally distributed, the non-parametric Wilcoxon signed-rank test was used. To investigate whether acetylcarnitine concentrations before the intervention (or other characteristics such as age and BMI) were predictive of the response to the intervention, a multivariable linear regression analysis was performed.



3 | RESULTS

3.1 | Participant characteristics

Thirty-two participants with well-controlled T2DM (HbA_{1c} of 6.6% [49.0 mmol/mol]) completed the study (21 men, 11 women). All participants were overweight to obese (BMI of 30.0 ± 1.0 kg/m^2) with an average age of 65 ± 1 years. The total overview of the participant characteristics is illustrated in Table S1. No dropouts or serious adverse events were reported. No major side effects of the oral carnitine supplementation were reported. Five participants experienced a slight fishy body odour, a recognized side effect of carnitine.

3.2 | Study compliance

As a sign of compliance, plasma-free carnitine (C0) as well as acetylcarnitine (C2) levels increased in all individuals upon carnitine supplementation ($p < 0.01$ for both, Figure 3A,B). All participants

FIGURE 3 Study compliance represented via plasma levels of free carnitine (A) and acetylcarnitine (B) concentrations ($n = 32$). Plasma samples were obtained in the overnight fasted state at baseline and month 3. Change in peripheral insulin sensitivity ($n = 30$) expressed as the insulin-stimulated rate of disappearance of glucose (ΔRd) during 40 mU/m^2/min of insulin infusion (C), insulin-stimulated non-oxidative glucose disposal ($n = 30$) during 40 mU/m^2/min of insulin infusion (D), substrate oxidation including carbohydrate (CHO) oxidation (E) and lipid oxidation (F), both $n = 30$, the percentage suppression of the endogenous glucose production (ΔEGP) as marker for hepatic insulin sensitivity ($n = 31$) with 10 mU/m^2/min insulin infusion (G), and fasting plasma glucose ($n = 32$) concentrations (H) are illustrate in the T2DM patients at baseline and month 3 after carnitine supplementation. MR-based skeletal muscle acetylcarnitine concentrations in the *m. vastus lateralis* in rest (pre-exercise) and the capacity to form acetylcarnitine with exercise (post-exercise) as marker of free carnitine availability (I) ($n = 32$) and Intrahepatic lipid content (J) ($n = 31$) determined using proton magnetic spectroscopy using a STEAM sequence are illustrated in type 2 diabetes patients before and after 3 months of carnitine supplementation. Data are presented as mean ± SEM and $n = 32$. C0: Free carnitine, C2: Acetylcarnitine. * Significantly different ($p < 0.05$), # trend towards a significant difference ($p < 0.10$).

TABLE 1 Body composition, physical activity level and plasma biochemistry at baseline and 3 months of carnitine supplementation.

Variable	Baseline	Month 3	Delta[b]	*p*-value
Body weight (kg)	89.41 ± 2.80	89.78 ± 2.80	0.37 ± 0.32	0.248
Fat-free mass (kg)	55.77 ± 1.75	56.12 ± 1.69	0.34 ± 0.40	0.392
Fat mass (kg)	33.63 ± 1.84	33.58 ± 1.96	−0.05 ± 0.45	0.909
Fat percentage (%)	37.20 ± 1.36	36.88 ± 1.42	−0.32 ± 0.48	0.517
Total physical activity score[a]	7.61 ± 0.26	7.73 ± 0.26	0.12 ± 0.11	0.278
Fasting glucose (mmol/L)	8.2 ± 0.3	7.7 ± 0.3	−0.43 ± 0.80	0.083
HbA_{1c} (mmol/mol)	48.7 ± 1.5	48.0 ± 1.5	−0.75 ± 0.80	0.357
HbA_{1c} (%)	6.6 ± 2.2	6.5 ± 2.2	−0.1 ± 0.80	0.357
Insulin (μU/mL)	7.8 ± 8.39	7.5 ± 7.9	−3.12 ± 5.90	0.601
Free fatty acids (μmol/L)	627.9 ± 49.3	603.5 ± 47.4	−24.4 ± 22.7	0.299[c]
Triglycerides (mmol/L)	1.9 ± 0.2	1.9 ± 0.2	0.06 ± 0.07	0.413
Cholesterol (mmol/L)	4.2 ± 0.2	4.3 ± 0.2	0.10 ± 0.10	0.287

Note: Data are presented as mean ± SEM and $n = 32$. HbA_{1c}, glycated haemoglobin.
[a]Total physical activity score assessed by Baecke's habitual physical activity questionnaire.
[b]Delta: month 6-baseline values.
[c]Data are not normally distributed and are non-parametrically tested.

returned the surplus of carnitine chewing tablets, and the compliance rate was calculated as the number of tablets ingested relative to the prescribed number. The compliance rate during the 3-month carnitine supplementation period was 97.1% ± 0.7%. In line with the instruction to maintain the usual lifestyle during participation in the study, no differences were observed in physical activity level ($p = 0.278$, Table 1) and body mass ($p = 0.248$) and composition (fat mass $p = 0.909$, fat-free mass $p = 0.392$, Table 1) after carnitine supplementation.

3.3 | Carnitine supplementation increased peripheral and hepatic insulin sensitivity

Peripheral insulin sensitivity was markedly improved by 31% after carnitine supplementation ($p = 0.005$, Figure 3C and Table 2). Elevation in insulin-stimulated non-oxidative glucose disposal ($\Delta NOGD_{high\text{-}basal}$) largely explained this increased peripheral insulin sensitivity ($p = 0.004$, Figure 3D and Table 2), whereas the insulin-stimulated glucose oxidation ($\Delta CHO_{high\text{-}basal}$) remained unaffected by carnitine supplementation ($p = 0.866$, Table 2). Absolute glucose oxidation tended to be higher in all stages of the hyperinsulinemic-euglycemic clamp after carnitine supplementation but only reached statistical significance in the low-insulin state ($p = 0.018$, Figure 3E and Table 2). Lipid oxidation showed a reciprocal pattern and tended to be lower after carnitine supplementation in all stages of the clamp. Similarly, statistical significance was reached in the low-insulin state ($p = 0.004$, Figure 3F and Table 2) while it remained a tendency towards significance during the high-insulin state ($p = 0.063$, Figure 3F and Table 2). Metabolic flexibility was not different in the high insulin phase ($p = 0.710$) but improved in the low insulin phase upon carnitine supplementation ($p = 0.007$, Table 2). Interestingly, hepatic insulin sensitivity (ΔEGP suppression) strongly improved by 22% from 32.4 ± 3.0 to 39.5 ± 3.2%; $p = 0.020$, Figure 3G. Adipose tissue insulin sensitivity, calculated as the suppression of FFA levels during the low-insulin state, was not affected by carnitine supplementation (from 58.9 ± 2.9% to 59.4 ± 3.9%, $p = 0.95$).

3.4 | Fasting plasma glucose levels tended to be lower after carnitine supplementation

Fasting plasma glucose tended to be reduced after 3 months of carnitine supplementation ($p = 0.083$, Figure 3H and Table 1) while basal EGP was not significantly altered by carnitine supplementation ($p = 0.138$). These reduced plasma glucose levels did not translate into lowered HbA_{1c} nor insulin concentration ($p = 0.357$ and $p = 0.601$ respectively, Table 1). In addition, no changes in plasma-free fatty acids, triglycerides and cholesterol were observed upon carnitine supplementation (Table 1). Plasma short-, medium- and long-chain acylcarnitines were all elevated ($p < 0.001$, Table S2).

3.5 | Enhanced skeletal muscle acetylcarnitine formation due to carnitine supplementation

To investigate whether improvements in skeletal muscle acetylcarnitine metabolism contributed to the enhanced peripheral insulin sensitivity and substrate oxidation after carnitine supplementation, acetylcarnitine concentrations were measured in vivo in the m. *vastus lateralis* using ^{1}H-MRS before and after exercise. Indeed, resting skeletal muscle acetylcarnitine concentration increased after carnitine supplementation (1.18 ± 0.13 vs. 1.54 ± 0.17 mmol/kgww; $p = 0.004$, Figure 3I). Skeletal muscle acetylcarnitine levels were determined after 30 min of exercise at 70% W_{max}. Exercise is a strong stimulator of skeletal muscle acetylcarnitine formation,[9,19] possibly because

TABLE 2 Insulin sensitivity and substrate kinetics during the two-step hyperinsulinemic-euglycemic clamp.

	Baseline	Month 3	Delta[a]	*p*-value
Rd glucose (μmol × kg^{-1} × min^{-1})				
Basal	11.73 ± 0.53	10.96 ± 0.53	−0.77 ± 0.50	0.136
10 mU/m^2/min	10.51 ± 0.33	11.02 ± 0.50	0.51 ± 0.38	0.190
40 mU/m^2/min	22.26 ± 1.62	24.80 ± 1.83	2.54 ± 1.05	0.022
Δ10-basal	−1.22 ± 0.52	0.05 ± 0.64	1.28 ± 0.61	0.046
Δ40-basal	10.53 ± 1.85	13.83 ± 2.02	3.31 ± 1.10	0.005
EGP (μmol × kg^{-1} × min^{-1})				
Basal	11.36 ± 0.52	10.75 ± 0.46	−0.61 ± 0.40	0.138
10 mU/m^2/min	7.59 ± 0.37	6.51 ± 0.42	−1.08 ± 0.28	0.001
40 mU/m^2/min	2.04 ± 0.26	1.63 ± 0.22	−0.41 ± 0.19	0.045
Δ10-basal	−3.77 ± 0.39	−4.24 ± 0.36	−0.47 ± 0.42	0.269
Δ40-basal	−9.32 ± 0.50	−9.12 ± 0.35	0.20 ± 0.47	0.671
NOGD (μmol × kg^{-1} × min^{-1})				
Basal	9.87 ± 0.78	8.47 ± 0.60	−1.40 ± 0.48	0.007
10 mU/m^2/min	5.81 ± 0.47	4.90 ± 0.70	−0.91 ± 0.62	0.153
40 mU/m^2/min	13.56 ± 1.35	15.55 ± 1.53	1.99 ± 1.04	0.066
Δ10-basal	−4.05 ± 0.54	−3.56 ± 0.72	0.49 ± 0.72	0.505
Δ40-basal	3.69 ± 1.56	7.08 ± 1.60	3.39 ± 1.08	0.004
CHO oxidation (μmol × kg^{-1} × min^{-1})				
Basal	1.86 ± 0.60	2.49 ± 0.47	0.63 ± 0.50	0.217
10 mU/m^2/min	4.69 ± 0.43	6.11 ± 0.60	1.42 ± 0.56	0.018
40 mU/m^2/min	8.69 ± 0.61	9.24 ± 0.71	0.55 ± 0.51	0.294
Δ10-basal	2.83 ± 0.43	3.62 ± 0.49	0.79 ± 0.31	0.017
Δ40-basal	6.83 ± 0.65	6.75 ± 0.58	−0.08 ± 0.49	0.866
Lipid oxidation (μmol × kg^{-1} × min^{-1})				
Basal	1.26 ± 0.05	1.21 ± 0.04	−0.05 ± 0.04	0.182
10 mU/m^2/min	1.00 ± 0.04	0.86 ± 0.05	−0.13 ± 0.04	0.004
40 mU/m^2/min	0.72 ± 0.04	0.64 ± 0.05	−0.08 ± 0.04	0.063
Δ10-basal	−0.27 ± 0.04	−0.35 ± 0.04	−0.08 ± 0.03	0.011
Δ40-basal	−0.55 ± 0.05	−0.57 ± 0.04	−0.02 ± 0.04	0.618
RER (arbitrary units AU)				
Basal	0.746 ± 0.009	0.755 ± 0.006	0.008 ± 0.007	0.228
10 mU/m^2/min	0.787 ± 0.006	0.810 ± 0.008	0.022 ± 0.008	0.011
40 mU/m^2/min	0.841 ± 0.007	0.852 ± 0.008	0.011 ± 0.007	0.106
Δ10-basal	0.041 ± 0.006	0.055 ± 0.008	0.014 ± 0.005	0.007
Δ40-basal	0.095 ± 0.009	0.097 ± 0.008	0.003 ± 0.007	0.710

Note: Data are presented as mean ± SEM.
Abbreviations: CHO, carbohydrate oxidation; EGP, endogenous glucose production; NOGD, non-oxidative glucose disposal; Rd., rate of disappearance.
[a]Delta: month 3-baseline values.

mitochondrial substrate flux rapidly increases. This eventually leads to elevated intra-mitochondrial acetyl-CoA levels and concomitant increased acetylation of carnitine to acetylcarnitine. Therefore, at high exercise intensity, acetylcarnitine concentration strongly increases, and the total pool of free carnitine is almost completely acetylated. Under these conditions, acetylcarnitine concentration therefore reflects free carnitine availability. Indeed, post-exercise acetylcarnitine concentrations increased after carnitine supplementation (3.70 ± 0.22 vs. 4.53 ± 0.30 mmol/kgww; $p < 0.001$, Figure 3I) suggesting carnitine supplementation improves free carnitine availability. Furthermore, we investigated whether patient characteristics (such as age, BMI and carnitine status (high-low levels prior to the start of the study)) could be predictive for the individual improvement in insulin sensitivity in the current study. However, none of the investigated characteristics

were associated with the response in insulin sensitivity in the current study ($p > 0.05$).

3.6 | Tendency towards reduced intrahepatic lipid content

Intrahepatic lipid content (IHL) was determined in vivo using ^{1}H-MRS to investigate if a reduction in IHL could have contributed to the improvement in hepatic insulin sensitivity. Interestingly, liver fat content tended to be reduced after carnitine supplementation (from 14.7 to 12.8 ± 2.2%; $p = 0.098$, Figure 3J).

3.7 | Carnitine supplementation effects on NAD metabolism in skeletal muscle

Metabolomics revealed a significant decrease in creatinine and nicotinamide levels, and a tendency for a decrease in lactate levels in skeletal muscle after 3 months of carnitine supplementation. No other metabolites were affected by carnitine supplementation (Figure S4). However, the significant differences disappeared after FDR correction.

3.8 | Improvement in daily living, physical performance and cognitive function after carnitine supplementation

Daily physical performance was improved in patients with T2DM after carnitine supplementation, as illustrated by an increased walking distance during the 6MWT ($p < 0.01$, Table S3) and reduced stand-sitting time determined via the TCST ($p < 0.01$, Table S3). Carnitine supplementation did not affect QoL nor QoS ($p = 0.609$ and $p = 0.720$ respectively, Table S3). Cognitive performance on the psychomotor speed function motor screening task (MOT) was improved after carnitine supplementation, as indicated by a decrease in mean latency from stimulus onset to the correct response to that stimulus (LM) ($p = 0.042$, Table S3). No alterations were present in the reaction time (RT) task. The executive functions of multitasking (MTT) and spatial span (SSP) improved after carnitine supplementation. This carnitine-driven benefit in MTT was indicated by a tendency towards a smaller multitasking cost (MCT) ($p = 0.096$, Table S3) as well as a substantial decrease in the number of incorrect answers ($p = 0.002$, Table S3). Spatial span (SSP) tended to be elevated, as indicated by a higher forward span length (FSL) meaning a longer sequence of boxes successfully recalled ($p = 0.093$, Table S3). Memory, represented via paired associated learning (PAL), improved after 3 months of carnitine supplementation, as illustrated by a higher number of times a participant chose the correct box on the first attempt when recalling pattern locations (FAMS) ($p = 0.024$, Table S3). Furthermore, a trend towards a lower total number of errors ($p = 0.104$, Table S3) was reported. No difference in the memory DMS test was observed.

4 | DISCUSSION

Acetylcarnitine has gained attention as an important metabolite in light of maintaining insulin sensitivity and glucose homeostasis.[3–6] Free carnitine availability is suggested to be crucial in the formation of acetylcarnitine, and indeed, animal studies revealed a link between reduced free carnitine availability and metabolic disease.[5,6,8] In accordance with our hypothesis, we here show that 3 months of carnitine supplementation increases free carnitine availability in plasma and acetylcarnitine in muscle and concomitantly profoundly improves peripheral as well as hepatic insulin sensitivity. Therefore, carnitine supplementation might be an interesting addition to conventional medication to promote insulin sensitivity in T2DM.

Free carnitine availability was previously reported to be reduced in patients with T2DM.[37] We here show that 3 months of carnitine supplementation elevates plasma-free carnitine concentration. In addition, we investigated if skeletal muscle acetylcarnitine also increased. Interestingly, a notable increase in resting skeletal muscle acetylcarnitine concentration as well as the capacity to form acetylcarnitine with exercise was observed after carnitine supplementation in patients with type 2 diabetes, indicating that the supplemented carnitine reaches skeletal muscle and increases carnitine availability. These results are in line with our previous research in pre-diabetes individuals[9] where acetylcarnitine in muscle was increased after an even shorter period of supplementation. Together, these data support the notion that the capacity to form acetylcarnitine may be limited by the availability of free carnitine[4,6] and can be restored by supplementation.

We previously showed that nicotinamide riboside supplementation for 6 weeks in healthy volunteers with obesity increased resting skeletal muscle acetylcarnitine levels, which have been suggested to be markers of metabolic flexibility and insulin sensitivity.[32] Interestingly, a study in obese mice showed that the combination of supplementation of NR with l-carnitine led to a reduction in FM percentage and hepatic steatosis.[38] Here, we did not find major effects of carnitine supplementation on NAD metabolism. Therefore, the exact interaction between carnitine and NAD metabolism – if any – requires further investigation.

We postulated that increasing free carnitine availability enhances the capacity to form acetylcarnitine, which in turn improves insulin sensitivity. In line with our hypothesis, 3 months of carnitine supplementation profoundly increased peripheral insulin sensitivity by 31% in patients with type 2 diabetes. Similar results on markers of insulin sensitivity (HOMA-IR) were reported.[10–12,14,39] However, here we used the gold standard hyperinsulinemic-euglycemic clamp and show that the improvement in insulin sensitivity in the current study is mainly explained by insulin-stimulated NOGD rather than glucose oxidation. Similar improvements in NOGD were previously reported after carnitine infusion during a hyperinsulinemic-euglycemic clamp in patients with T2DM.[40,41] Glycogen synthesis in skeletal muscle tissue is known to be impaired in patients with type 2 diabetes, caused by a reduced stimulating effect of insulin on glycogen synthase.[42] These observations suggest that carnitine may stimulate insulin-stimulated

glycogen synthesis, which is also supported by some in vitro results in the liver[43]; however, data on skeletal muscle is yet lacking. Regarding substrate oxidation, we did observe improvement in metabolic flexibility upon carnitine supplementation, consistent with our previous finding,[9] but this effect was only observed in the low insulin phase of the clamp, although RER also tended to be increased upon carnitine supplementation during high insulin infusion. No effect of carnitine supplementation on fat oxidation was observed, in contrast to our previous finding.[9] Carnitine has been suggested to stimulate fat oxidation, but most studies have investigated the effect of carnitine on substrate oxidation during exercise[19,44] and mainly in healthy volunteers. It is likely that the effects of carnitine supplementation in patients with type 2 diabetes, who are characterized by disturbed metabolic regulation, are different from those in healthy volunteers, but comparative studies are needed to understand the lack of effect of fat oxidation in patients with type 2 diabetes.[38]

Besides peripheral insulin sensitivity, we investigated for the first time the effect of 3 months of carnitine supplementation on hepatic insulin sensitivity in patients with T2DM. Interestingly, hepatic insulin sensitivity improved by 22% after carnitine supplementation. Since hepatic insulin resistance is associated with increased ectopic lipid deposition in the liver,[45] we investigated whether a reduced IHL content is underlying the improvements in hepatic insulin sensitivity after carnitine supplementation. Indeed, a tendency towards lower IHL was found in type 2 diabetes supplemented with carnitine. Next to potentially increasing fat oxidation in the liver,[46] free carnitine can scavenge acyl chains in the liver and export these as acylcarnitines to the blood. Studies investigating trans-organ acylcarnitine fluxes revealed that plasma acylcarnitines mainly reflect hepatic acylcarnitine export.[47,48] In line, we here reported elevated plasma concentrations of acetylcarnitine, medium- and long-chain acylcarnitines.

In line with the results on insulin sensitivity, we reported a tendency towards lower fasting plasma glucose levels in patients with type 2 diabetes after carnitine supplementation. These lower fasting plasma glucose levels are consistent with Rahbar et al.,[13] who also reported lower fasting plasma glucose levels after carnitine supplementation. The lack of statistical significance in the current study could be explained by the supplementation duration, as Asadi et al. recently revealed that a minimum of 12 weeks of carnitine supplementation is needed to establish a reduction in fasting plasma glucose levels.[39] In patients with type 2 diabetes, enhanced gluconeogenesis rates contribute to elevated fasting plasma glucose levels.[49] Previously, it was reported that carnitine supplementation reduces gluconeogenesis in animals[50] which may potentially explain the tendency for lower fasting plasma glucose found here. Therefore, adding carnitine supplementation to the conventional treatment of patients with T2DM might be a good strategy to reduce hyperglycaemia, highlighting the clinical relevance of carnitine supplementation. The current study did not show improvements in HbA_{1c} levels. Because the HbA1c measurement comprises a 3-month average of glycated haemoglobin, it is likely that the supplementation duration of 3 months in the current study is too short to induce changes. Indeed, studies with similar or shorter supplementation durations failed to improve HbA_{1c},[13,51,52] whilst reductions in HbA_{1c} are reported with longer study durations (48 weeks).[11,39,53]

Lastly, we explored whether the improvements in insulin sensitivity in these patients with type 2 diabetes could translate into benefits in daily life functioning. Indeed, we found some indications of improved physical and cognitive health. These findings suggest that carnitine supplementation does not only beneficially affect metabolic health but may also improve daily life functioning, though longer and larger studies are needed to confirm these results.

A limitation of our study is the absence of a placebo-controlled group. Study-induced changes in energy intake and physical activity are prone to placebo effects. Therefore, we monitored these factors but did not report any changes after 3 months of carnitine supplementation, implicating no contribution of lifestyle change to a possible placebo effect. Nevertheless, to estimate the magnitude of a possible placebo effect on insulin sensitivity, we determined the changes in the control groups in various interventional studies with similar designs and similar participant populations (patients with type 2 diabetes).[54–57] In these studies, a maximum placebo-induced effect of +8% on insulin sensitivity was reported in the placebo-controlled groups. In the current study, the reported effect of carnitine supplementation on insulin sensitivity is very pronounced, reaching a 31% increase. Therefore, we are convinced that the pronounced effect on insulin sensitivity in the current study indeed originates from the administered carnitine. The absence of a control group might be most problematic for the exploratively investigated parameters reported in Table S3 (such as 6MWT and TCST tests). However, it should be noted that when comparing the effect of carnitine to the effect of placebo supplementation as reported earlier, 50%–100% stronger improvements were found in the current study.[58] Therefore, we believe that carnitine supplementation improves daily life functioning, though longer and larger studies including a classic control are needed to confirm these results. Finally, it should be noted that in the current study, a carnitine dose of 3000 mg/day was used, which is considered high. Although we did not observe any major side effects in the current investigation, it has been suggested that long-term L-carnitine use could raise levels of trimethylamine-N-oxide (TMAO) over time, and TMAO has been associated with an enhanced risk of atherosclerosis.[59] Long-term studies with carnitine supplementation in humans are needed to investigate the side effects of high-dose carnitine supplementation.

5 | CONCLUSION

Three months of carnitine supplementation (2970 mg/day) improved peripheral insulin sensitivity by 31% in patients with overweight/obesity and type 2 diabetes and tended to reduce fasting plasma glucose levels. In addition, hepatic insulin sensitivity was enhanced, accompanied by improved metabolic flexibility at low insulin infusion rates and a tendency towards lower intrahepatic lipid content. Increased free carnitine availability in skeletal muscle (reflected by the ability to form acetylcarnitine with exercise) might be underlying these

profound effects on metabolic health. Finally, daily life functioning (i.e. physical and cognitive performance) improved upon carnitine supplementation, indicating patients also perceived benefits from the applied intervention. However, future research including a classical placebo-controlled trial is necessary. In conclusion, we demonstrated that daily carnitine supplementation might be an interesting add-on therapy in patients with type 2 diabetes.

AUTHOR CONTRIBUTIONS

Y.M.H.B., R.B.B., A.V., M.K.C.H., P.S. and V.B.S.-H. contributed to the conceptualization of the study. The methodology was developed by Y.M.H.B., E.P., M.K.C.H., P.S. and V.B.S.-H. Formal analysis was conducted by Y.M.H.B., R.P., P.V., G.S. and E.K., while the investigation was carried out by Y.M.H.B., Y.J.M.K. and B.H. Y.M.H.B. was responsible for writing the original draft, and the review and editing were undertaken by Y.J.M.K., R.H., P.V., E.P., B.H., G.S., R.B.B., A.V., J.E.W., M.K.C.H., P.S. and V.B.S.-H. Supervision was provided by V.B.S.-H., J.E.W., M.K.C.H. and P.S. Project administration was managed by Y.M.H.B., V.B.S. and P.S., while funding acquisition was handled by V.B.S.-H.

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CONFLICT OF INTEREST STATEMENT

B.R.B and A.V. are employees of Alfasigma Nederland B.V.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Esther Phielix https://orcid.org/0000-0002-2672-0415

Vera B. Schrauwen-Hinderling https://orcid.org/0000-0001-7795-584X

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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