Research paper

Carnitine supplementation improves metabolic flexibility and skeletal muscle acetylcarnitine formation in volunteers with impaired glucose tolerance: A randomised controlled trial

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\textbf{A B S T R A C T}

\textbf{Background:} Type 2 diabetes patients and individuals at risk of developing diabetes are characterized by metabolic inflexibility and disturbed glucose homeostasis. Low carnitine availability may contribute to metabolic inflexibility and impaired glucose tolerance. Here, we investigated whether carnitine supplementation improves metabolic flexibility and insulin sensitivity in impaired glucose tolerant (IGT) volunteers.

\textbf{Methods:} Eleven IGT- volunteers followed a 36-day placebo- and L-carnitine treatment (2g/day) in a randomised, placebo-controlled, double blind crossover design. A hyperinsulinemic-euglycemic clamp (40 mU/m²/min), combined with indirect calorimetry (ventilated hood) was performed to determine insulin sensitivity and metabolic flexibility. Furthermore, metabolic flexibility was assessed in response to a high-energy meal. Skeletal muscle acetylcarnitine concentrations were measured in vivo using long echo time proton magnetic resonance spectroscopy (\textsuperscript{1}H-MRS, TE=500 ms) in the resting state (7:00 AM and 5:00 PM) and after a 30-min cycling exercise. Twelve normal glucose tolerant (NGT) volunteers were included without any intervention as control group.

\textbf{Results:} Metabolic flexibility of IGT-subjects completely restored towards NGT control values upon carnitine supplementation, measured during a hyperinsulinemic-euglycemic clamp and meal test. In muscle, carnitine supplementation enhanced the increase in resting acetylcarnitine concentrations over the day (delta 7:00 AM versus 5:00 PM) in IGT-subjects. Furthermore, carnitine supplementation increased post-exercise acetylcarnitine concentrations and reduced long-chain acylcarnitine species in IGT-subjects, suggesting the stimulation of a more complete fat oxidation in muscle. Whole-body insulin sensitivity was not affected.

\textbf{Conclusion:} Carnitine supplementation improves acetylcarnitine formation and rescues metabolic flexibility in IGT-subjects. Future research should investigate the potential of carnitine in prevention/treatment of type 2 diabetes.

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1. Introduction

Metabolic flexibility is defined as the capacity to switch from predominantly fat oxidation while fasting, to carbohydrate oxidation in the insulin-stimulated state as defined by Kelley and...
Mandarino [1]. Rates of fat- and carbohydrate oxidation can be measured by determining whole body oxygen consumption and carbon dioxide production by indirect calorimetry. Performing indirect calorimetry measurements in the fasted and insulin stimulated state thereby allows assessment of metabolic flexibility. Decreased metabolic flexibility is an early hallmark in the development of type 2 diabetes mellitus. Impairments in metabolic flexibility are not only present in patients with type 2 diabetes but also in a pre-diabetic state, in individuals with so-called impaired glucose tolerance (IGT) [2]. Decreased metabolic flexibility in these individuals results in delayed postprandial glucose clearance and thereby eventually leads to disturbances in glucose homeostasis. Therefore, improving metabolic flexibility in people with IGT may be a good strategy to delay, mitigate and/or prevent disturbed glucose homeostasis.

It has recently been suggested that the formation of acetyl carnitine is crucial in maintaining metabolic flexibility, resulting in improved glucose homeostasis [3–6]. Acetyl carnitine is synthesized by the conjugation of acetyl-CoA and free carnitine, mediated via the enzyme carnitine acetyltransferase (CrAT) [3–6]. This enzyme acts as a buffering system to prevent extreme fluctuations in acetyl-CoA and free CoA availability. Buffeting of the acetyl and free CoA pools is especially important during physiological conditions that cause substantial shifts in mitochondrial substrate supply and demand, such as (over)feeding and exercise. Acetyl-CoA is an allosteric regulator of mitochondrial enzymes and high levels of acetyl-CoA have been linked to states of metabolic inertia. This may be caused by nutrient overabundance which can lead to conflicting signals controlling mitochondrial substrate traffic and selection. Thus, the inflow of acetyl-CoA from fatty acid oxidation can override signaling from insulin to switch from lipid catabolism to glucose use, resulting in metabolic inflexibility and a muted response to a carbohydrate meal [6]. Conversely, sequestration of free CoA might limit maximal rates of β-oxidation during fasting. In accordance, knocking-out the CrAT enzyme in mice results in lower acetyl carnitine formation and blunted metabolic flexibility during feeding and exercise. Furthermore, CrAT gain-of-function studies in human primary myotubes showed elevated mitochondrial acetyl carnitine efflux, indicating enhanced acetyl carnitine formation with higher CrAT enzyme activity [3], resulting in higher pyruvate dehydrogenase (PDH) activity and metabolic flexibility [3]. These results suggest an important role for acetyl carnitine formation in maintaining metabolic flexibility.

When lipid supply to the mitochondria exceeds demand and/or enzymatic capacity, a mismatch between β-oxidation and TCA cycle flux can result in accumulation of β-oxidation intermediates, such as long chain acyl-CoA and long chain acylcarnitine species [6,7]. Indeed, elevated long-chain acylcarnitines were reported in insulin resistance and in conditions of blunted metabolic flexibility [8]. The ‘trapping’ of free carnitine and free CoA due to accumulation of β-oxidation intermediates, such as long chain acyl-CoA may limit acylcarnitine synthesis, as free carnitine availability is crucial for maintaining the buffering capacity of CrAT [3,9]. Therefore, next to low CrAT activity, decreased carnitine availability may also contribute to the development of metabolic inflexibility. For example, it was found that diabetic mice (beta actin promotor (BAP) agouti transgenic mice) were characterized by reduced acetyl carnitine concentrations in muscle, which could be restored to levels of non-diabetic mice by carnitine supplementation [9]. This was accompanied by improved metabolic flexibility, insulin sensitivity and restoration of blood glucose levels [9]. Furthermore, Noland et al. [4] found that feeding rats a chronic high-fat diet decreased the availability of free carnitine and hampered metabolic flexibility. Again, supplementation of carnitine in these rats restored metabolic flexibility and insulin sensitivity [4].

Whether carnitine supplementation can also improve metabolic flexibility in humans has so far not been studied. Moreover, it still remains elusive whether the beneficial effects of carnitine supplementation on metabolic flexibility and concomitantly improved glucose tolerance in humans relates to enhanced capacity to form acetyl carnitine. Intervention trials in humans have shown that oral carnitine supplementation can improve glucose tolerance in insulin resistant individuals with low carnitine status [10–12] and can have beneficial effects on glucose levels [13,14], insulin levels [12] and markers of insulin resistance, such as the homeostatic model assessment of insulin resistance (HOMA-IR) index [12,13] or glucose area under the curve after an oral glucose tolerance test [15]. These results seem to suggest that carnitine supplementation may enhance the capacity to form acetyl carnitine and improve metabolic flexibility in humans. To facilitate examination of this hypothesis, we recently set up a novel, proton magnetic resonance spectroscopy (1H-MRS) protocol, using long echo times, to repeatedly determine acetyl carnitine concentrations in skeletal muscle in vivo [16]. This technique provides the unique opportunity to non-invasively and dynamically investigate the role of acetyl carnitine formation in the development of metabolic inflexibility and type 2 diabetes. Using this non-invasive approach, we here aimed to investigate in humans if carnitine supplementation leads to increased intramuscular acetyl carnitine formation while also improving metabolic flexibility and insulin sensitivity in individuals with impaired glucose tolerance.

2. Methods

2.1. Ethical approval

The study protocol was approved by the institutional Medical Ethical Committee and conducted in accordance with the declaration of Helsinki. Monitoring was performed by the Clinical Trial Center of Maastricht. The study was registered at clinicaltrials.gov with identifier NCT02072759.

2.2. Participants

Twelve normal glucose tolerant (NGT) and eleven impaired glucose tolerant (IGT) individuals were included. IGT was defined as a plasma glucose level between 7.8–11.1 mmol/L 2-h after an oral glucose tolerance test (OGTT) and NGT as plasma glucose levels <7.8 mmol/L two hours after OGTT [4,17]. Exclusion criteria were unstable body weight (weight gain or loss >3 kg in the previous 3 months), engagement in exercise >3 h a week, impaired renal and/or kidney function, uncontrolled hypertension, history of cardiovascular disease, MRI contra-indications and being vegetarian. Impairments in renal and/or kidney function were assessed via determination of plasma ASAT, ALAT, GGT, creatine and bilirubin. An electrocardiogram (ECG) and blood pressure measurement were performed to assess cardiovascular diseases and uncontrolled hypertension respectively. All screening measurements were evaluated by a medical doctor. All individuals gave written informed consent.

2.3. Experimental design and treatment

The study was set up in a double blind, randomised cross-over design in volunteers with IGT. NGT volunteers served as control group for baseline comparison (Fig. 1A). The study was conducted at Maastricht University, The Netherlands, between April 2014 and June 2016. IGT participants were randomly assigned [18] to two intervention periods: 36 days oral intake of placebo and carnitine (2000 mg/day of l-Carnitine tartrate (NOW foods, Blooming-
dalen, IL, USA). The carnitine dosage was based on previous studies in humans reporting improvements in metabolic parameters after 2000 mg of oral carnitine supplementation [10,13,15,19–21]. Participants consumed one 500-mg capsule in the morning during breakfast, one 500-mg capsule at noon during lunch and two 500-mg capsules in the evening during dinner. In the morning of test-day 30 and 33, subjects did not take any supplements. During participation in the study, both NGT- and IGT-subjects were instructed to maintain their habitual diet and physical activity pattern. Furthermore, intake of food supplements was not allowed to prevent additional intake of oral carnitine. Three days before the test days, participants refrained from strenuous physical activity. Wash-out was at least 4 weeks. Compliance was checked counting unused supplements and by measuring plasma acylcarnitines levels using tandem mass spectroscopy on day 0 and 33 [22]. Participants were asked to maintain their normal diet and physical activity pattern during participation in the study. Participants reported to the laboratory on a weekly basis (day 0,7,14,21,28,33) for a fasting blood sample, to check compliance and supply supplements for the next week. On day 30, 33 and 36 various measurements were performed as described below. NGT participants were subjected to the same measurements but without any intervention trial (no placebo and carnitine). Primary outcome was the effect of L-carnitine supplementation on metabolic flexibility and insulin sensitivity. Secondary outcome measures were acetylcarnitine formation and plasma and skeletal muscle acylcarnitine profiles.

2.4. VO2max and body composition

Before the start of the trial, participants underwent an incremental cycling test to determine maximal oxygen uptake (VO2max) and maximal power output (Wmax) for characterization of the participants. On the same day, body composition was determined by DXA (DXA, discovery A; Hologic).

2.5. Meal test

On day 30, participants reported to the laboratory at 6:00 AM after an overnight fast and in vivo skeletal muscle acetylcarnitine concentrations were determined by 1H-MRS (see below). Subsequently, a meal test was performed (Fig. 1B). To this end, a high-energy breakfast was provided to the participants at 8:00 AM (t = 0), which consisted of sausage rolls (65.5% energy from fat, 31.6% energy of carbohydrates, and 6.9% energy form proteins, total energy content was equal to 50% of the participants required daily energy intake, according to the Cunningham equation [23] with a correction factor of 1.3 for physical activity). Blood plasma was sampled repeatedly to measure plasma glucose, insulin, free fatty acids and triglycerides (at t = −45, t = 15, t = 150, t = 330 min). Before the meal, and at t = 90 min, and t = 270 min, indirect calorimetry measurements (Ventilated hood, Omnicl, Maastricht Instruments, Maastricht University) were performed. The respiratory exchange ratio (RER) was used to determine metabolic flexibility (ΔRERt-90–fasted and ΔRERt-270–t = 90) and substrate oxidation 1.5 h (t = 90 min) and 4.5 h (t = 270 min) after consumption of the breakfast.

2.6. Hyperinsulinemic-euglycemic clamp

On day 33, a one-step hyperinsulinemic-euglycemic clamp was performed to assess peripheral insulin sensitivity [24]. Subjects reported to the laboratory at 7:30 AM after an overnight fast. A fasted blood sample was taken and subsequently a primed-continuous infusion of D-[6,6-2H2]-glucose (16.8 mg/mL, 0.04 ml/kg/min) was started to determine rates of glucose appearance (Rg), glucose disappearance (Ra) and endogenous glucose production (EGP) [25]. After 2 h (t = 120 min), infusion of insulin (40 mU/m2/min) was started for 2.5 h. During the basal period (t = 90–120 min) and the last 30 min of insulin infusion (t = 240–270 min) blood samples were collected and indirect calorimetry
(ventilated hood) was performed to assess metabolic flexibility and substrate utilization according to Peronnet et al. [26]. Isotopic enrichment of plasma glucose was determined by electron ionization gas chromatography-mass spectrometry as described previously [27]. Steele's single pool non-steady state equations were used to calculate glucose $R_0$ and $R_1$ [25]. Volume of distribution was assumed to be 0.160L/kg for glucose.

2.7. Muscle biopsy

On the morning of the hyperinsulinemic euglycemic clamp, a muscle biopsy was taken from the vastus lateralis muscle according to the Bergström method [28]. Muscle tissue was frozen in melting isopentane and stored at −80°C until further processing. Skeletal muscle acylcarnitines were analysed as previously described using electrospray tandem mass spectrometry [29]. Glycogen concentration was determined by using a commercial glycogen assay kit (ab65620, Abcam, Cambridge, United Kingdom) according to instructions of the manufacturer.

2.8. Cycling test

Finally, on a third test day (day 36), participants reported to the laboratory at 4:30 PM. After consumption of a light lunch at 12:00 AM, participants remained fasted for the following 5 h and refrained from physical activity. After arrival at the laboratory, subjects rested for 30 min. At 5:00 PM, baseline skeletal muscle acylcarnitine concentrations were measured by $^1$H-MRS. Subsequently, a 30-min cycling exercise at 70% of the participants pre-determined maximal power output ($W_{max}$) was performed on an ergometer in a room next to the MR scanner. Directly after the exercise, quantification of acylcarnitine was repeated.

2.9. $^1$H-MRS (acylcarnitine)

Acylcarnitine was quantified as reported earlier on a 3T clinical MR scanner (Achieva 3T-X, Philips Healthcare, Best, The Netherlands [16]). Spectra were acquired with the following acquisition parameters: TR=6000, spectral bandwidth 2kHz and number of acquired data points 2048. A series of spectra were acquired with variable TE and NSA (300–12, 325–16, 350–20, 400–32, 450–52, 500–76 respectively). Due to considerable lipid contamination, acylcarnitine concentration was analysed in spectra with TE=500ms as the shorter echo times showed considerable lipid contamination in overweight participants. Using this method, the detection limit is approximately 0.15 mM.

Baseline correction was performed for all acquired spectra with a custom-made MATLAB script (The Mathworks Inc.). Spectra were analysed using the AMARES algorithm in JMRUI software [30]. The creatine resonance (t-Cr) was used as internal reference and acylcarnitine concentration was calculated assuming a creatine concentration of 30mmol/kg ww. TE corrections were performed for creatine (T2=166ms) and acylcarnitine (T2=262ms) and a correction for the dipolar coupling of creatine was applied and set at 30% of the signal as reported earlier [16].

2.10. Sample size and statistics

The effect of oral supplementation of carnitine on insulin sensitivity as determined by the clamp technique, has not been investigated yet. Therefore, the sample size was calculated based on the results from carnitine infusion studies which show clinically significant improvements of about 9.4% in parameters of insulin sensitivity during a hyperinsulinemic-euglycemic [31,32]. The individual variation (SD) of the difference in insulin sensitivity in repeated measurements is reported to be around 10% [24,33,34]. To reach 80% power and a significance level of 0.05 (two-sided) a minimal calculated sample size of N=11 was needed. Data are presented as means ± SEM. Statistical analysis were performed using SPSS 24.0 software (SPSS, Chicago, IL.). Results were considered significantly different when p<0.05 (two-sided testing). To evaluate if the data were normally distributed a Shapiro-Wilk normality test was performed. Student’s paired t-test were performed to compare the intervention trials (carnitine and placebo). NGT and IGT participants were compared by Student’s independent sample t-tests. Potential carry-over effect between treatment and period as well as period effect were examined by unpaired t-test analyses according to Pocock et al. [35]. No carry-over or period effects were found. Using individual data, Pearson correlation coefficients were calculated to test for associations between parameters. A mixed model repeated measures ANOVA was conducted to investigate changes in meal-induced metabolic flexibility, plasma metabolites (C0 and C2) and skeletal muscle acylcarnitine concentrations assessed by $^1$H-MRS. Likewise, plasma metabolites before, during and after 30 days of carnitine and placebo treatment were assessed using a mixed model repeated measures ANOVA. Post-hoc analysis were performed and Bonferroni correction was applied to correct for multiple testing.

3. Results

3.1. Subject characteristics

Body composition and maximal oxygen uptake ($VO_2$max) were comparable between groups. Fasting glucose levels were slightly higher in IGT participants (p=0.034) and, by design, glucose levels 120 min after an oral glucose tolerance test (OGTT) were higher in IGT (p<0.01). Also insulin levels at t=120 min of the OGTT were significantly different between groups (p=0.01). Fasting insulin, cholesterol and triglyceride levels were similar across groups at the onset of the study. Baseline characteristics are reported in Table 1. Carnitine supplementation did not alter fasting plasma glucose, insulin, cholesterol, triglycerides and liver function (Table 2).

| Table 1  | Baseline participant characteristics. |
|----------|---------------------------------------|
|          | NGT (n = 12) | IGT (n = 11) |
| Sex (m/f) | 10 / 2       | 10 / 1       |
| Age (y)    | 61 ± 2       | 62 ± 2       |
| BMI (kg/m²)| 28.9 ± 0.7   | 29.7 ± 0.5   |
| Body composition | | |
| Fat mass (kg) | 26.8 ± 1.8 | 27.0 ± 1.1 |
| Fat free mass (kg) | 64.8 ± 2.5 | 64.2 ± 2.9 |
| Fat percentage (%) | 29.6 ± 1.9 | 29.8 ± 1.0 |
| Visceral adipose tissue (kg) | 0.6 ± 0.1 | 0.8 ± 0.1 |
| Physical fitness | | |
| VO2max (ml/min·1·kg⁻¹) | 29.7 ± 1.5 | 28.3 ± 1.4 |
| Wmax (W·kg⁻¹) | 2.2 ± 0.1 | 2.3 ± 0.1 |
| Oral glucose tolerance test (OGTT) | | |
| Fasting glucose (mmol/L) | 5.3 ± 0.1 | 5.7 ± 0.1 |
| Glucose OGTT20 (mmol/L) | 4.6 ± 0.3 | 8.2 ± 0.3 |
| Fasting insulin (pmol/L) | 56.6 ± 9.1 | 80.1 ± 14.2 |
| Insulin OGTT20 (pmol/L) | 223.5 ± 36.1 | 669.3 ± 149.3 |
| Blood lipid profile | | |
| Total cholesterol (mmol/L) | 5.6 ± 0.2 | 5.3 ± 0.1 |
| HDL cholesterol (mmol/L) | 1.5 ± 0.2 | 1.3 ± 0.1 |
| LDL cholesterol (mmol/L) | 3.4 ± 0.2 | 3.2 ± 0.2 |
| Triglycerides (mmol/L) | 1.64 ± 0.36 | 1.76 ± 0.28 |

p < 0.05, IGT significantly different from NGT. Wmax, maximal workload.Data are represented as mean ± SEM.
Table 2
Plasma metabolites before and after 30 days of carnitine and placebo.

|                  | IGT placebo | IGT carnitine | P-value |
|------------------|-------------|---------------|---------|
| Glucose (mmol/L) |              |               |         |
| Day 0            | 5.7 ± 0.2   | 5.6 ± 0.2     | 0.315   |
| Day 7            | 5.5 ± 0.1   | 5.6 ± 0.2     |         |
| Day 14           | 5.4 ± 0.1   | 5.5 ± 0.2     |         |
| Day 21           | 5.4 ± 0.5   | 5.9 ± 0.2     |         |
| Day 28           | 5.6 ± 0.2   | 5.6 ± 0.2     |         |
| Day 33           | 5.4 ± 0.1   | 5.6 ± 0.2     |         |
| Insulin (pmol/L) |              |               |         |
| Day 0            | 68.8 ± 9.0  | 78.6 ± 11.7   | 0.501   |
| Day 7            | 75.0 ± 13.7 | 71.8 ± 13.2   |         |
| Day 14           | 72.8 ± 10.6 | 88.0 ± 17.0   |         |
| Day 21           | 89.5 ± 17.5 | 114.9 ± 21.1  |         |
| Day 28           | 46.2 ± 6.4  | 58.2 ± 9.8    |         |
| Day 33           | 56.2 ± 9.3  | 59.6 ± 10.5   |         |
| HbA1C (%)        |              |               |         |
| Day 0            | 5.5 ± 0.1   | 5.5 ± 0.1     | 0.921   |
| Day 33           | 5.5 ± 0.1   | 5.6 ± 0.1     |         |
| Blood lipid profile |            |               |         |
| Total cholesterol (mmol/L) | 5.7 ± 0.3 | 5.9 ± 0.2 | 0.521 |
| Day 0            | 5.7 ± 0.3   | 5.8 ± 0.2     |         |
| Day 7            | 5.6 ± 0.3   | 6.0 ± 0.3     |         |
| Day 14           | 5.5 ± 0.2   | 5.7 ± 0.2     |         |
| Day 21           | 5.6 ± 0.3   | 5.1 ± 0.3     |         |
| Day 28           | 5.4 ± 0.2   | 5.5 ± 0.3     |         |
| Day 33           |              |               |         |
| HDL cholesterol (mmol/L) | 1.4 ± 0.1 | 1.4 ± 0.1 | 0.391 |
| Day 0            | 1.4 ± 0.1   | 1.4 ± 0.2     |         |
| Day 7            | 1.2 ± 0.1   | 1.4 ± 0.2     |         |
| Day 14           | 1.4 ± 0.1   | 1.2 ± 0.1     |         |
| Day 21           | 1.6 ± 0.2   | 1.1 ± 0.1     |         |
| Day 28           | 1.3 ± 0.1   | 1.3 ± 0.1     |         |
| Day 33           |              |               |         |
| LDL cholesterol (mmol/L) | 3.4 ± 0.3 | 3.6 ± 0.2 | 0.620 |
| Day 0            | 3.5 ± 0.3   | 3.6 ± 0.3     |         |
| Day 7            | 3.5 ± 0.3   | 3.5 ± 0.2     |         |
| Day 14           | 3.3 ± 0.3   | 3.3 ± 0.2     |         |
| Day 21           | 3.3 ± 0.1   | 2.9 ± 0.2     |         |
| Day 28           | 3.2 ± 0.2   | 3.2 ± 0.2     |         |
| Day 33           |              |               |         |
| Triglycerides (mmol/L) | 2.1 ± 0.3 | 2.6 ± 0.5 | 0.512 |
| Day 0            | 2.1 ± 0.3   | 2.6 ± 0.5     |         |
| Day 7            | 2.5 ± 0.5   | 3.6 ± 1.5     |         |
| Day 14           | 2.9 ± 0.5   | 3.0 ± 0.4     |         |
| Day 21           | 2.6 ± 0.3   | 2.2 ± 0.4     |         |
| Day 28           | 2.0 ± 0.2   | 2.1 ± 0.3     |         |
| Day 33           |              |               |         |
| Liver function |               |               |         |
| ASAT (U/L)       | 27.1 ± 2.3  | 25.2 ± 2.0    | 0.678   |
| Day 33           | 23.7 ± 1.5  | 23.6 ± 3.4    |         |
| ALAT (U/L)       | 28.1 ± 3.6  | 29.8 ± 3.4    | 0.758   |
| Day 33           | 25.6 ± 2.7  | 32.3 ± 4.5    |         |
| Gamma-GT (U/L)   | 39.2 ± 8.2  | 39.4 ± 6.3    | 0.546   |
| Day 33           | 34.6 ± 6.0  | 32.3 ± 6.3    |         |

Data are expressed as mean ± SEM. P-value reflect time×treatment effect by mixed model repeated measures ANOVA. Bonferroni correction was performed to correct of multiple testing. Plasma values are obtained after an overnight fast. There were no missing data in these parameters.

3.2. Elevations in plasma free carnitine and acetylcarnitine levels

A time×group interaction was found for plasma free carnitine (p<0.001) and acetylcarnitine concentrations (p=0.021). Plasma free carnitine (C0) and acetylcarnitine (C2) concentrations were similar at the beginning of placebo and carnitine treatment (p=0.99 and p=0.687 respectively). Upon carnitine supplementation, plasma free carnitine as well as acetylcarnitine concentrations increased in all individuals (C0 from 40.8±1.6 to 50.5±1.7 μmol/L, p<0.001 and C2 from 6.6±0.4 to 8.3±0.6 μmol/L, p=0.034, Fig. 2A and B), indicating compliance to the study protocol. Carnitine levels did not change after placebo treatment (C0 from 39.4±1.4 to 39.8±1.3 μmol/L, p=0.770 and C2 from 6.0±0.4 to 6.2±0.3 μmol/L, p=0.538, Fig. 2A and B). No major side effect of the oral carnitine supplementation were reported. Only one IGT subject experienced a slightly fishy body odor.

3.3. Carnitine supplementation restored metabolic flexibility but not insulin sensitivity during a hyperinsulinemic-euglycemic clamp

Metabolic flexibility, expressed as the change in respiratory exchange ratio upon insulin stimulation (ΔER), was significantly lower in IGT compared to NGT participants (Fig. 2D, p=0.022). Interestingly, carnitine supplementation completely restored metabolic flexibility to NGT values in IGT-subjects (Fig. 2D, p=0.01). Overnight fasted lipid and carbohydrate oxidation rates were not different between NGT and IGT. However, carnitine supplementation increased overnight fasted basal lipid oxidation compared to placebo (p=0.034), resulting in fat oxidation levels that were higher than in normal glucose tolerant participants (Fig. 2F, p=0.007). In line with the notion that fat- and carbohydrate oxidation rates generally exhibit reciprocal trends, basal carbohydrate oxidation was reduced on carnitine supplementation compared to the placebo group (p=0.013) and tended to be lower in comparison with NGT participants (Fig. 2E, p=0.055). Substrate oxidation in the insulin-stimulated state was similar in the placebo and carnitine supplemented condition. Therefore, the change in metabolic flexibility (ΔER) is driven by a change in basal substrate oxidation. Together with the reduction in carbohydrate oxidation in the fasting state in IGT participants upon carnitine supplementation, non-oxidative glucose disposal in the fasted state was increased (p<0.05) in these volunteers, indicating that a larger fraction of the glucose taken up was incorporated into glycogen.

We also examined if carnitine supplementation had beneficial effects on peripheral insulin sensitivity and performed a hyperinsulinemic euglycemic clamp combined with deuterated glucose tracers. Basal EGP was lower in IGT participants compared to NGT (p<0.01) but was not affected by carnitine supplementation (p=0.962). Insulin-stimulated suppression of EGP was not significantly different between groups (p<0.05).

As expected, the insulin-stimulated rate of disappearance of glucose (ΔRg) was significantly higher in NGT compared to IGT participants (p<0.01, Table 4). Carnitine supplementation did not significantly affect insulin stimulated glucose disposal (ΔRg: 11.74±1.99 vs 13.32±3.08 after placebo and carnitine respectively, p=0.512). Interestingly, however, upon carnitine supplementation, a larger fraction of this insulin stimulated glucose disposal was directed towards glucose oxidation, thereby completely restoring the insulin-stimulated glucose oxidation in IGT towards levels observed in NGT (Table 4). Conversely, carnitine supplementation reduced insulin-stimulated non-oxidative glucose disposal. These data suggest that, although carnitine supplementation did not affect peripheral insulin sensitivity, it did result in a redistribution of the glucose taken up after insulin stimulation towards oxidative disposal and less to glycogen storage.

3.4. Skeletal muscle glycogen concentrations

To directly test if carnitine supplementation affected baseline muscle glycogen concentrations, we measured glycogen concentration in the muscle biopsies. Muscle glycogen was not different between NGT and IGT participants (p=0.05) in the fasted state. Although not significant, skeletal muscle glycogen concentrations tended to increase upon carnitine supplementation (p=0.140,
3. Metabolic flexibility is rescued in IGT-subjects during a high-energy meal test upon carnitine supplementation

Next to the assessment of metabolic flexibility during a clamp, metabolic flexibility was also determined in a more physiological setting, upon a meal. In line with the results from the clamps, metabolic flexibility in response to a high-energy meal was also decreased in IGT and could be completely restored with carnitine supplementation (time x group interaction p<0.05, Fig. 2G-I). IGT participants on carnitine supplementation had a lower RER in the fasted state compared to placebo (p=0.024) as well as when compared to NGT controls (p=0.027). Although metabolic flexibility improved, meal-induced levels of plasma glucose, insulin, triacylglycerides and free fatty acids were unaffected by carnitine supplementation (time x group interaction p>0.05, Fig. 3).

3.6. Carnitine supplementation improves capacity to form acetylcarnitine in muscle

To investigate if the improved metabolic flexibility upon carnitine supplementation resulted in improved acetylcarnitine metabolism, we used 1H-MRS to determine acetylcarnitine levels in the vastus lateralis muscle. We first determined if carnitine supplementation would elevate overnight fasted acetyl-carnitine levels. Contrary to our expectations, skeletal muscle acetylcarnitine concentrations in the morning at 7:00 AM were not significantly different between NGT and IGT (0.37±0.09 vs 0.65±0.15 mmol/kgww respectively, p=0.111), and if anything tended to be elevated in IGT. Also, carnitine supplementation did not alter muscle acetylcarnitine concentrations (0.44±0.12 mmol/kgww for carnitine, p=0.674) as measured by 1H-MRS. These data match with the acetylcarnitine concentrations measured in biopsies where likewise, no difference between the groups or between the placebo and carnitine treatment were detected (see data below). Given the hypothesized role of acetylcarnitine in metabolic flexibility, it can be expected that these levels are low in the overnight fasted state when the body mainly relies on fat as a substrate source. We therefore also measured acetylcarnitine levels later during the day. Interestingly, skeletal muscle acetylcarnitine levels were higher when measured at
5:00 PM as compared to 7:00 AM in NGT (Fig. 4A and B), suggesting that acetyl carnitine levels in skeletal muscle rise during the day. Furthermore, a time * group interaction was found (p = 0.028). Intriguingly, this increase in skeletal muscle acetyl carnitine levels during the day (assessed in a subset of 8 participants) was markedly blunted in the IGT group (delta acetyl carnitine concentration: 0.67 ± 0.18 vs 1.41 ± 0.33 μmol/kgww in IGT compared to NGT, p = 0.048, Fig. 4A and B), but was completely restored upon carnitine supplementation (1.51 ± 0.33 mmol/kgww, p = 0.037). As a result, acetyl carnitine levels measured at 5:00 PM tended to be lower in IGT participants on placebo compared to NGT controls (1.08 ± 0.20, 1.64 ± 0.28 mmol/kgww for IGT and NGT respectively, p = 0.064) and in fact, carnitine supplementation restored acetyl carnitine levels towards values observed in NGT (1.62 ± 0.27 mmol/kgww).

Finally, we also determined the maximal capacity to form acetyl carnitine. Exercise is known to lead to an increase in skeletal muscle acetyl carnitine, possibly because substrate load into the mitochondria is rapidly increased upon exercise thereby elevating acetyl-CoA levels. At high exercise intensity, this increase in acetyl carnitine reflects the capacity individuals have to produce acetyl carnitine and can be seen as a parameter for free carnitine availability. Therefore, acetyl carnitine was measured in skeletal muscle before exercise (5:00 PM measurement) and after 30 min of exercise at 70% W_max. Acetyl carnitine levels in skeletal muscle increased in all three groups (p < 0.05), but post-exercise acetyl carnitine concentrations were markedly higher after carnitine supplementation compared to placebo (4.23 ± 0.53 vs 3.60 ± 0.49 mmol/kgww for carnitine and placebo respectively, p = 0.017) and reached NGT-levels (4.15 ± 0.28 mmol/kgww, Fig. 4C).
Table 4
Substrate kinetics and insulin sensitivity.

| RER (arbitrary units AU) | NGT (n = 12) | IGT Placebo (n = 11) | IGT Carnitine (n = 11) |
|--------------------------|--------------|----------------------|------------------------|
| Basal                    | 0.798 ± 0.008| 0.791 ± 0.012        | 0.762 ± 0.010<sup>a,b</sup> |
| Insulin-stimulated       | 0.899 ± 0.009| 0.857 ± 0.011<sup>a</sup> | 0.865 ± 0.010<sup>b</sup> |
| Δ                        | 0.101 ± 0.012| 0.066 ± 0.007<sup>a</sup> | 0.100 ± 0.010<sup>b</sup> |
| CHO oxidation (μmol·kg<sup>-1</sup>·min<sup>-1</sup>) | | | |
| Basal                    | 6.71 ± 0.61  | 5.93 ± 0.71          | 4.01 ± 0.66<sup>b</sup> |
| Insulin-stimulated       | 14.22 ± 0.78 | 10.77 ± 0.75         | 11.09 ± 0.79<sup>a</sup> |
| Δ                        | 7.51 ± 1.01  | 4.84 ± 0.48          | 7.08 ± 0.74<sup>a</sup> |
| Lipid oxidation (μmol·kg<sup>-1</sup>·min<sup>-1</sup>) | | | |
| Basal                    | 1.10 ± 0.05  | 1.12 ± 0.08          | 1.27 ± 0.07<sup>a,b</sup> |
| Insulin-stimulated       | 0.55 ± 0.05  | 0.77 ± 0.07          | 0.73 ± 0.06<sup>b</sup> |
| Δ                        | −0.54 ± 0.07 | −0.35 ± 0.04         | −0.54 ± 0.05<sup>b</sup> |
| Rd glucose (μmol·kg<sup>-1</sup>·min<sup>-1</sup>) | | | |
| Basal                    | 10.80 ± 0.70 | 7.64 ± 0.49<sup>a</sup> | 7.97 ± 0.80<sup>b</sup> |
| Insulin-stimulated       | 36.51 ± 3.48 | 19.38 ± 2.02<sup>a</sup> | 21.29 ± 2.98<sup>a</sup> |
| Δ                        | 25.71 ± 3.39 | 11.74 ± 1.99<sup>a</sup> | 13.32 ± 3.08<sup>a</sup> |
| NOGD (μmol·kg<sup>-1</sup>·min<sup>-1</sup>) | | | |
| Basal                    | 5.41 ± 0.70  | 3.09 ± 0.49<sup>a</sup> | 5.94 ± 0.80<sup>b</sup> |
| Insulin-stimulated       | 23.16 ± 3.48 | 10.98 ± 2.02<sup>a</sup> | 9.08 ± 2.98<sup>a</sup> |
| Δ                        | 17.76 ± 3.39 | 7.89 ± 1.99<sup>a</sup> | 3.15 ± 3.08<sup>a</sup> |
| Glucose (mmol/L)          | | | |
| Basal                    | 5.5 ± 0.1    | 5.8 ± 0.1<sup>a</sup> | 5.9 ± 0.1<sup>a</sup> |
| Insulin-stimulated       | 5.5 ± 0.2    | 5.3 ± 0.1            | 5.2 ± 0.1 |
| Plasma insulin (pmol/L)  | | | |
| Basal                    | 63.85 ± 11.48| 84.31 ± 9.16         | 84.24 ± 11.48 |
| Insulin-stimulated       | 945.07 ± 52.39| 1022.01 ± 43.04     | 981.08 ± 5.56 |
| Plasma FFA (μmol/L)      | | | |
| Basal                    | 522.86 ± 30.96| 612.00 ± 56.68       | 597.4 ± 34.75 |
| Insulin-stimulated       | 72.31 ± 6.61 | 119.90 ± 14.02<sup>a</sup> | 110.30 ± 11.28<sup>a</sup> |

<sup>a</sup> IGT placebo or IGT carnitine significantly different from NGT.
<sup>b</sup> IGT carnitine significantly different from IGT placebo. Data are expressed as mean ± SEM.

Fig. 3. Plasma metabolites during the meal test, glucose (A), free fatty acids FFA (B), triglycerides (C), insulin (D) analysed via a mixed model repeated measures ANOVA. No interaction effects (time × group) were present for any of the plasma metabolites. The black line represents the NGT group, the striped line the IGT on placebo and the dotted line IGT on carnitine. Data are expressed as means ± SEM. * significantly different (p < 0.05). Values refer to twelve participants in the NGT group and nine participants in the IGT P and IGT C groups. In two IGT participants, the meal test could not be performed due to technical problems.
suggesting that carnitine supplementation boosted the maximal capacity to form acetylcarnitine.

3.7. Lower whole body RER upon carnitine supplementation is related to more complete degradation of fat substrates

A limitation of MRS is that only acetyl carnitine levels can be measured and no information is provided about other acyl carnitine species. Therefore, we examined the complete acyl carnitine profile in the muscle biopsies taken after an overnight fast after placebo or carnitine supplementation. As described above, over the whole group, concentrations of free carnitine (C0), acetyl carnitine (C2) and other short-chain acyl carnitine species were similar in muscle biopsies from NGT and IGT subjects (for C0: p = 0.416 and for C2: p = 0.935), and unaffected by carnitine supplementation (for C0: p = 0.356 and for C2: p = 0.371). However, there were some inter-individual differences and the subjects who showed the greatest change in carnitine availability upon supplementation also showed the greatest response in fat oxidation. Thus, the change in skeletal muscle free carnitine availability from placebo to carnitine treatment period correlated positively with the change in lipid oxidation in the fastest state (r = 0.66, p = 0.03, Fig. 5A). Furthermore, the change in skeletal muscle acetyl carnitine concentrations also correlated positively with the change in lipid oxidation in the fastest state (r = 0.63, p = 0.04, Fig. 5B).

Long-chain acyl carnitines were elevated in IGT on placebo compared to NGT (p = 0.047), suggesting less complete β-oxidation in IGT volunteers. Interestingly, carnitine supplementation actually lowered long-chain acyl carnitine species compared to placebo, (p = 0.027, Fig. 5D, Table 5). This observation suggests carnitine supplementation contributed to more complete β-oxidation. To gain further insight into changes in complete fat oxidation, we calculated the acetyl carnitine (C2)/long-chain acyl carnitine ratio. Interestingly, the individuals that increased their fat oxidation the most also showed the greatest changes in the C2 acetyl carnitine/long-chain acyl carnitine ratio, suggesting that the lower whole body RER upon carnitine supplementation is due to more complete degradation of fat substrates (Fig. 5C).

4. Discussion

Animal studies have indicated that free carnitine availability in skeletal muscle may be crucial in permitting the formation of acetyl carnitine and coincident maintenance of metabolic flexibility, as indicated by Noland et al. [4]. We here aimed to investi-
gate substrate for carnitine palmitoyl transferase 1 (CPT1), which synthesizes long chain acylcarnitines at the outer mitochondrial membrane and thereby permits transfer of long chain acyl groups into the matrix. Thus, diminished CPT1 activity due enzyme inhibition and/or carnitine insufficiency could limit rates of fat oxidation. However, as muscle carnitine concentrations are in far excess of the in vitro Michaelis Menten constant of CPT1 in human muscle, carnitine is generally not assumed to be limiting to CPT1 flux [36]. Nevertheless, some studies investigated the impact of carnitine on substrate oxidation and maximal performance in trained athletes and found that fat oxidation during exercise was increased and maximal performance improved. However, this was found indeed without affecting CPT1 activity [37,38], arguing against increased CPT1 flux underlying the changes found at carnitine supplementation. Nevertheless discussion remains on whether compartmentalization may play a role and that specifically mitochondrial carnitine concentration may be of relevance [39]. In the current study, we find improvements of whole body fat oxidation in the resting state, and also here it is unlikely that an increased CPT1 flux is underlying our findings, as the elevated fat oxidation in the current study is not linked to accumulation of long chain acyl-carnitines. The accumulation of long-chain acylcarnitines in skeletal muscle has previously been reported in situations when high lipid availability drives fat oxidation. Under such circumstances, long chain acylcarnitine production by CPT1 surpasses downstream capacity of the beta-oxidation enzymes, resulting in incomplete fat oxidation and potential trapping of both carnitine and CoA moieties [4,6,7]. In the current study, the rise in whole-body fat oxidation that occurred in response to carnitine supplementation was accompanied by decreased levels of skeletal muscle long-chain acylcarnitines, strongly suggesting that CPT1 flux was not the limiting factor during the placebo arm of the trial. Instead, the findings imply that carnitine supplementation improved fat oxidative capacity by promoting acyl-CoA flux through the entire beta-oxidation spiral, resulting in more complete degradation of long chain fatty acids to acetyl-CoA and CO2.

Although some studies were performed on the effect of carnitine on substrate metabolism during exercise and maximal performance, to our knowledge, it has not been investigated whether carnitine can beneficially influence substrate oxidation in the resting state in humans and whether it can thereby affect the meal-induced increment of carbohydrate oxidation. Here, we investigated this in a highly relevant population of volunteers with impaired glucose tolerance and therefore at increased risk of developing type 2 diabetes and found that indeed, carnitine supplementation resulted in a more pronounced switch between fat oxidation in the fasted state and carbohydrate oxidation in the insulin-stimulated state based on the RQ data measured with indirect

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**Fig. 5. Lower whole body RER upon carnitine supplementation is related to more complete degradation of fat substrates.** Difference in skeletal muscle free carnitine availability (C0 in mmol/kgww) and acetylcarnitine (C2 in mmol/kgww) between placebo and carnitine treatment, measured in biopsies. In A, basal free carnitine availability is related to the lipid oxidation in the fasted state (r = 0.66, p = 0.03), figure 8). In B, the basal acetylcarnitine concentration is related to the lipid oxidation (R0) in the fasted state (r = 0.63, p = 0.04). In C, C2 acetylcarnitine/Long-chain acyl carnitine ratio is related to the lipid oxidation in the fasted state (r = 068, p = 0.02). In D, skeletal muscle long-chain acylcarnitine concentrations measured in biopsies are represented. Black bars represent the NGT group, white bars the IGT on placebo and grey bars IGT on carnitine. Data are expressed as means ± SEM. * IGT on carnitine significantly from IGT placebo (p=0.05), # IGT on carnitine tending to be different from IGT on placebo (p=0.10), † IGT placebo tending to be different from NGT (p=0.10).
calorimetry. The importance of diurnal changes in substrate oxidation and the beneficial metabolic effects of reaching a truly fasted state with strong reliance on fat oxidation is becoming increasingly recognized [40].

Despite the effects of carnitine supplementation on skeletal muscle acylcarnitine concentration and metabolic flexibility, no changes in insulin sensitivity were observed in the current study. Although the current study cannot reveal the reason for this lack of effect on insulin sensitivity, previous results suggest that the duration of the carnitine supplementation may have been too short to improve insulin sensitivity. Thus, Gonzalez-Ortiz et al. [41] and Galloway et al. [42] did not report changes in glucose homeostasis after four and two weeks of 3 g L-carnitine supplementation respectively. Indeed, changes in fasting plasma glucose and insulin, as well as improved HOMA-IR indices were observed upon 12, 24 and 48 weeks of oral L-carnitine supplementation suggesting that the duration of 36 days as in our study might be too short to improve insulin sensitivity. Future studies are therefore needed to reveal if longer duration of carnitine supplementation would result in improved insulin sensitivity.

A limitation of the current study is the small number of female subjects included (n = 3). Although no restrictions were applied for females participating in the study, recruitment of females was very difficult, resulting in a majority of male subjects. Even though the response to carnitine in females did not appear different than in male participants in the current study, the number of subjects is too low to draw definitive conclusions and future research will have to investigate in more detail whether the results can be translated to a female population.

In conclusion, we here show that carnitine supplementation has very pronounced effects on metabolic flexibility in impaired glucose tolerant volunteers, and in fact can completely restore metabolic flexibility. Carnitine supplementation enhanced the increase in acylcarnitine concentration in resting muscle throughout the day, as well as the capacity to form acylcarnitine in response to exercise. These changes in acylcarnitine formation may be underlying the beneficial effects on metabolic flexibility. Longer studies are needed to investigate if carnitine supplementation can also improve insulin sensitivity. Taken together, carnitine supplementation may be an interesting aid in improving disturbed metabolism in subjects prone to develop type 2 diabetes mellitus.

**Declaration of Competing Interest**

The authors declare no competing interest.

**CRediT authorship contribution statement**

Yvonne MH Bruls: Methodology, Formal analysis, Investigation, Writing - original draft, Project administration. Marlies de Ligt: Investigation, Writing - review & editing. Lucas Lindeboom: Methodology, Writing - review & editing. Esther Phielix: Methodology, Writing - review & editing. Bas Havekes: Investigation, Writing - review & editing. Gert Schaart: Formal analysis. Esther Kornips: Formal analysis, Joachim E Wildberger: Writing - review & editing, Supervision. Matthijs KC Hesselink: Conceptualization, Methodology, Writing - review & editing, Supervision. Deborah Muolo: Conceptualization, Writing - review & editing.
Schauren: Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration. Vera B Schauren-Hinderling: Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Research in context

Type 2 diabetes patients and individuals at risk of developing diabetes are characterized by metabolic inflexibility and disturbed glucose homeostasis. Low carnitine availability may contribute to metabolic inflexibility and impaired glucose tolerance as suggested based on animal studies. Whether carnitine supplementation can also improve metabolic flexibility in humans has so far not been studied. Therefore, we investigated whether carnitine supplementation improves metabolic flexibility and insulin sensitivity in impaired glucose tolerant (IGT) volunteers.

Added value of this study

Metabolic flexibility was completely restored upon carnitine supplementation in IGT volunteers. Furthermore, carnitine enhanced the increase in resting skeletal muscle acetylcarnitine concentrations over the day (delta 7:00 AM-5:00 PM). Long-chain acylcarnitine species were reduced after carnitine supplementation, suggesting the stimulation of a more complete fat oxidation in muscle.

Implication of all the available evidence

Carnitine supplementation may provide new opportunities in the prevention and/or treatment of type 2 diabetes which require further investigation.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2019.10.017.

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