Acetylcarnitine

Citation for published version (APA):

Bruls, Y. (2018). *Acetylcarnitine: The role in metabolic exibility and insulin sensitivity*. [Doctoral Thesis, Maastricht University]. Gildeprint Drukkerijen. <https://doi.org/10.26481/dis.20180614yb>

Document status and date:

Published: 01/01/2018

DOI:

[10.26481/dis.20180614yb](https://doi.org/10.26481/dis.20180614yb)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

ACETYLCARNITINE

The role in metabolic flexibility and insulin sensitivity

The studies presented in this thesis were performed within the framework of the NUTRIM School of Nutrition and Translational Research in Metabolism.

Financial support by Alfasigma Nederland BV for the publication of this thesis is gratefully acknowledged.

Cover design: Evelien Jagtman - evelienjagtman.com

Layout: Roger Bruls

Printed by: Gildeprint - gildeprint.nl

ISBN: 978-94-6233-973-6

© Y.M.H. Bruls, 2018, Maastricht, The Netherlands

No parts of this thesis may be reproduced, stored in a retrieval system, or transmitted in any form or by any means without the permission of the author, or, when appropriate, from the publishers of the manuscript.

ACETYLCARNITINE

The role in metabolic flexibility and insulin sensitivity

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof.dr. Rianne M. Letschert
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
donderdag 14 juni 2018 om 14.00 uur

door

Yvonne Maria Helena Bruls

Geboren op 27 juli 1990 te Heerlen

Promotores

Prof. dr. P. Schrauwen

Prof. dr. J.E. Wildberger

Copromotor

Dr. V.B. Schrauwen-Hinderling

Beoordelingscommissie

Prof. dr. R.P. Mensink (voorzitter)

Prof. dr. ir. A.H. Kersten (Universiteit Wageningen)

Prof. dr. S.W.M. Olde Damink

Prof. dr. R Shiri-Sverdlow

Dr. M. Soeters (AMC Amsterdam)

Table of contents

Chapter 1	General Introduction	7
Chapter 2	Isocaloric high-fat feeding decreases metabolic flexibility without alterations in insulin sensitivity and acylcarnitines species	17
Chapter 3	L-carnitine infusion does not alleviate lipid-induced insulin resistance and metabolic inflexibility	47
Chapter 4	Carnitine supplementation improves metabolic flexibility and skeletal muscle acetylcarnitine formation	81
Chapter 5	Longitudinal relaxation time editing for acetylcarnitine detection with ¹ H-MRS	117
Chapter 6	Resveratrol improves mitochondrial function but does not affect insulin sensitivity or brown adipose tissue activity in first degree relatives of patients with type 2 diabetes	135
Chapter 7	General discussion	167
Addendum	Summary	182
	Samenvatting	186
	Valorization	191
	Dankwoord	196
	List of publications	198
	About the author	199

Chapter 1

General introduction

Type 2 diabetes mellitus

The number of people with overweight is increasing rapidly worldwide, reaching pandemic proportions. A combination of excessive food intake (high-caloric, high-fat) and low physical activity are the primary contributors to the development of overweight (1). Overweight is associated with the development of chronic metabolic diseases such as type 2 diabetes mellitus (T2DM). An important characteristic of T2DM is that the metabolic effects of insulin on glucose metabolism are blunted (2). In the healthy state, the plasma concentration of glucose is tightly regulated within a narrow range. After food intake, the plasma concentration of the hormone insulin raises, causing the uptake of glucose into tissue, thereby regulating plasma glucose levels. In T2DM, this regulation is poor and hyperglycemia can develop. In the etiology of type 2 diabetes, different stages can be defined, i.e. normal glucose tolerance (NGT), impaired glucose tolerance (IGT), and finally T2DM. In normal glucose tolerant subjects, plasma glucose levels are <7.8 mmol/L when measured 2 hours after a 75 gram oral glucose bolus (OGTT). IGT is then defined as a plasma glucose level between 7.8-11.1 mmol/L 2-hours after OGTT. Further progression towards T2DM is defined by 2-hour OGTT values that exceed 11.1 mmol/L or fasting plasma glucose levels exceeding 7.0 mmol/L according to the world health organization (3, 4). The prevalence of T2DM is increasing dramatically worldwide. Recent estimates reported 171 million people worldwide diagnosed with T2DM in the year 2000 which is expected to increase towards 366 million in 2030 (5). Since T2DM is associated with reduced quality of life, decreased life expectancy, and increased risk of morbidities such as cardiovascular diseases, the diabetes-related costs are a major burden on our health care systems. Therefore, it is important to increase our understanding of this disease to improve prevention and cure.

The development of diabetes is also linked to changes in substrate oxidation. In the healthy condition, fat oxidation prevails in the fasted state and glucose oxidation is stimulated in the postprandial state. This variation in fat- and glucose oxidation is blunted in the diabetic condition, which is also termed metabolic inflexibility (2). Decreased metabolic flexibility is an early hallmark in the development of T2DM. Impairments in metabolic flexibility are not only present in patients with T2DM but also in a pre-diabetic state of impaired glucose tolerance (6).

Acetylcarnitine

Acetylcarnitine has recently been suggested as a very important metabolite in

maintaining metabolic flexibility, and subsequently glucose homeostasis and insulin sensitivity (4, 7-9). The formation of acetylcarnitine is mediated via the enzyme carnitine acetyltransferase (CrAT), which conjugates free carnitine and acetyl coenzyme A (acetyl-CoA), thereby releasing free CoA and maintaining intra-mitochondrial acetyl-CoA concentrations low (4, 7-9). Accumulation of intra-mitochondrial acetyl-CoA is known to hamper pyruvate-dehydrogenase (PDH) activity and thereby leads to compromised substrate switching and reduced glucose oxidation (7, 9-11). Therefore, acetylcarnitine formation from acetyl-CoA is suggested to be an important mitochondrial rescue mechanism to prevent intra-mitochondrial acetyl-CoA accumulation. Especially in conditions of substrate oversupply to the tricarboxylic acid (TCA) cycle, as during exercise or (over)feeding, production of acetyl-CoA may exceed the use by the TCA cycle, leading to intra-mitochondrial accumulation of acetyl-CoA. In these situations, formation of acetylcarnitine could avoid intra-mitochondrial accumulation of acetyl-CoA and prevent metabolic indecision. This metabolic indecision is referring to not choosing which substrate prevails for oxidation and thus metabolic inflexibility at the mitochondrial level develops (9). Therefore, CrAT-activity is very important in the formation of acetylcarnitine and metabolic flexibility. Indeed, animal models have indicated that knock-out of the CrAT enzyme in mice resulted in compromised metabolic flexibility and decreased acetylcarnitine formation (7). Furthermore, knock-out of the CrAT enzyme in these mice resulted in hampered glucose tolerance (7). Taken together, these findings suggest that acetylcarnitine formation is crucial in efficient mitochondrial substrate switching and maintaining glucose tolerance.

Free carnitine availability

Besides a properly functioning CrAT enzyme activity, the availability of free carnitine is also crucial in acetylcarnitine formation and maintaining metabolic flexibility (4, 7, 12). Free carnitine availability in rodents is reduced upon high-fat feeding compared to low-fat feeding resulting in reduced metabolic flexibility and finally insulin resistance (4, 10, 13, 14). Possibly, carnitine is trapped in the form of acyl-carnitines in these rodents. These acylcarnitines are intermediates of β -oxidation and accumulate, thereby reducing free carnitine availability (4, 10, 13, 14). In turn, this would reduce the formation of acetylcarnitine which may lead to hampered metabolic flexibility and insulin resistance as observed upon high-fat feeding. Also BAP-agouti transgenic mice (a mouse model for diabetes), characterized by blunted metabolic flexibility, insulin resistance and elevated blood glucose levels showed reduced levels of skeletal muscle free carnitine and

acetylcarnitine (12). Similar results of low free available carnitine were found in rats with compromised metabolic flexibility (4). Increasing free carnitine availability resulted in improved metabolic flexibility and insulin sensitivity in both animal models, providing evidence that sufficient availability of free carnitine is crucial in maintaining proper metabolic flexibility (4, 7, 12). This gave rise to the hypothesis that enhancing free carnitine in metabolic compromised humans might be a good strategy to combat metabolic inflexibility and insulin resistance by increasing the capacity to form acetylcarnitine. Possible strategies to enhance free carnitine availability could be intravenous infusion or oral supplementation of carnitine. Indeed, positive results of intravenous carnitine infusion in humans have been reported on markers of insulin resistance, such as glucose infusion rate (15, 16) and M-value (17), but it is unknown whether these positive effects can be attributed to enhanced acetylcarnitine formation. Also, oral carnitine supplementation in humans is reported to have positive effects on glucose homeostasis. Thus, after oral carnitine supplementation in humans, plasma glucose levels decreased (18, 19). As a consequence, insulin sensitivity, expressed as HOMA-IR index (16, 18) and glucose area under the curve after an oral glucose tolerance test (20) were improved. However, it is still unknown whether an increased capacity to form acetylcarnitine upon oral carnitine supplementation is underlying these improvements in insulin sensitivity and substrate switching leading to increased glucose tolerance.

In-vivo acetylcarnitine determination

Since the formation of acetylcarnitine might be very important for optimal regulated substrate switching and glucose tolerance, compromised acetylcarnitine formation might therefore play a crucial role in the etiology of T2DM (4, 7, 9, 10). To better understand this possible role of acetylcarnitine formation in substrate switching, insulin resistance and the etiology of T2DM, it is important to study skeletal muscle acetylcarnitine formation in humans. The common way of measuring acetylcarnitine in skeletal muscle tissue is via tandem-mass spectrometry in muscle biopsies (21-23). Recently, a novel non-invasive method using proton magnetic resonance spectroscopy (^1H -MRS) was developed in Maastricht (24). This MR based technique is using long echo times to determine acetylcarnitine concentration in skeletal muscle vastus lateralis *in vivo*. This novel MR based technique gives the opportunity to non-invasively and dynamically investigate the role of acetylcarnitine formation in the development of metabolic inflexibility, insulin resistance, and type 2 diabetes. Applying this novel and unique

technique revealed that type 2 diabetic patients had lower acetylcarnitine concentrations compared to trained athletes, suggesting a decreased acetylcarnitine formation with compromised mitochondrial status (24).

Thesis outline

This thesis mainly focuses on acetylcarnitine in skeletal muscle tissue and its role in metabolic flexibility and insulin sensitivity. In **chapter 2** we investigated the role of acylcarnitines in a human intervention that is expected to decrease mitochondrial function and metabolic flexibility. The effects of three weeks isoenergetic high-fat feeding on metabolic flexibility and insulin sensitivity in overweight male volunteers are described. High fat diet is known to decrease metabolic flexibility, and we therefore investigated if changes in free carnitine availability, acetylcarnitine, or other carnitine species could explain the earlier reported decrease in metabolic flexibility. Intravenous lipid infusion elevates plasma free fatty acid concentration and is a model for compromised insulin sensitivity and metabolic flexibility in healthy, insulin sensitive volunteers. In **chapter 3**, we aimed to investigate if acute carnitine infusion during simultaneous lipid infusion could alleviate lipid-induced insulin resistance and metabolic inflexibility in healthy young males by increasing free carnitine availability. Furthermore, free carnitine availability and other acylcarnitine species were investigated in skeletal muscle tissue. Next to the short-term elevation in carnitine availability by intravenous infusions, we also aimed to investigate the longer term effect upon a five-week intervention with oral carnitine supplementation. Therefore, **chapter 4** describes the results of a double-blind, randomized placebo-controlled trial investigating if oral carnitine supplementation improves metabolic flexibility and insulin resistance in volunteers with impaired glucose tolerance and whether this is mediated by increased formation of acetylcarnitine. In **chapter 5**, we developed an alternative Magnetic Resonance Spectroscopy (MRS) sequence to detect acetylcarnitine concentration *in vivo*. Previously, we reported that acetylcarnitine can be detected by MRS, using long echo times (TE=350 ms) as lipid resonances are suppressed due to T2 relaxation. In **chapter 3** and **4** we applied this MR technique. However, the spectra with TE=350 ms that were analyzed in **chapter 4** showed considerable lipid contamination in overweight participants (and therefore, even longer echo times were used for acetylcarnitine quantification in **chapter 4**). Thus, more pronounced T2-weighting proved necessary in this typical overweight population to determine acetylcarnitine concentrations at TE=350 ms. Therefore, a sequence that also makes use of the difference in T1 between

acetylcarnitine- and lipid resonances was developed. This new alternative methodology allows better distinguishing differences between lipid and acetylcarnitine, especially in subjects with high myocellular lipid concentrations such as overweight and type 2 diabetes patients. This allows more accurate quantification of acetylcarnitine concentrations at TE=350 ms in this population in future studies. In **chapter 6**, we investigated the role of acylcarnitines in a human intervention with resveratrol. Resveratrol has been shown to improve mitochondrial function and metabolic health in obese humans, and provides another model to investigate the relationship between acylcarnitines and metabolic flexibility in humans. Therefore, the effects of the food supplement resveratrol on insulin sensitivity, metabolic flexibility, mitochondrial function and skeletal muscle acylcarnitine species in impaired glucose tolerant subjects was investigated in **chapter 6**. Finally, in **chapter 7** the main results and conclusions of the previous chapters in the thesis are discussed in a broader perspective. Future directions for research within this field of metabolic research are addressed.

REFERENCES

1. Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128.9 million children, adolescents, and adults. *Lancet* (London, England). 2017.
2. Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes*. 2000;49(5):677-83.
3. WHO Expert Committee on Diabetes Mellitus: second report. World Health Organization technical report series. 1980;646:1-80.
4. Noland RC, Koves TR, Seiler SE, Lum H, Lust RM, Ilkayeva O, et al. Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control. *The Journal of biological chemistry*. 2009;284(34):22840-52.
5. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes care*. 2004;27(5):1047-53.
6. Faerch K, Vaag A. Metabolic inflexibility is a common feature of impaired fasting glycaemia and impaired glucose tolerance. *Acta diabetologica*. 2011;48(4):349-53.
7. Muoio DM, Noland RC, Kovalik JP, Seiler SE, Davies MN, DeBalsi KL, et al. Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. *Cell metabolism*. 2012;15(5):764-77.
8. Stephens FB, Constantin-Teodosiu D, Greenhaff PL. New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *The Journal of physiology*. 2007;581(Pt 2):431-44.
9. Muoio DM. Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. *Cell*. 2014;159(6):1253-62.
10. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell metabolism*. 2008;7(1):45-56.
11. Randle PJ, Garland PB, Hales CN, Newsholme EA. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* (London, England). 1963;1(7285):785-9.
12. Power RA, Hulver MW, Zhang JY, Dubois J, Marchand RM, Ilkayeva O, et al. Carnitine revisited: potential use as adjunctive treatment in diabetes. *Diabetologia*. 2007;50(4):824-32.

13. Wessels B, van den Broek NM, Ciapaite J, Houten SM, Wanders RJ, Nicolay K, et al. Carnitine supplementation in high-fat diet-fed rats does not ameliorate lipid-induced skeletal muscle mitochondrial dysfunction *in vivo*. *American journal of physiology Endocrinology and metabolism*. 2015;309(7):E670-8.
14. Wang ZQ, Zhang XH, Yu Y, Poulev A, Ribnicky D, Floyd ZE, et al. Bioactives from bitter melon enhance insulin signaling and modulate acyl carnitine content in skeletal muscle in high-fat diet-fed mice. *The Journal of nutritional biochemistry*. 2011;22(11):1064-73.
15. Capaldo B, Napoli R, Di Bonito P, Albano G, Sacca L. Carnitine improves peripheral glucose disposal in non-insulin-dependent diabetic patients. *Diabetes research and clinical practice*. 1991;14(3):191-5.
16. Molino A, Cascino A, Conte C, Ramaccini C, Rossi Fanelli F, Laviano A. Caloric restriction and L-carnitine administration improves insulin sensitivity in patients with impaired glucose metabolism. *JPEN Journal of parenteral and enteral nutrition*. 2010;34(3):295-9.
17. Giancaterini A, De Gaetano A, Mingrone G, Gniuli D, Liverani E, Capristo E, et al. Acetyl-L-carnitine infusion increases glucose disposal in type 2 diabetic patients. *Metabolism: clinical and experimental*. 2000;49(6):704-8.
18. Malaguarnera M, Gargante MP, Russo C, Antic T, Vacante M, Malaguarnera M, et al. L-carnitine supplementation to diet: a new tool in treatment of nonalcoholic steatohepatitis—a randomized and controlled clinical trial. *The American journal of gastroenterology*. 2010;105(6):1338-45.
19. Rahbar AR, Shakerhosseini R, Saadat N, Taleban F, Pordal A, Goldestan B. Effect of L-carnitine on plasma glycemic and lipidemic profile in patients with type II diabetes mellitus. *European journal of clinical nutrition*. 2005;59(4):592-6.
20. Ruggenenti P, Cattaneo D, Loriga G, Ledda F, Motterlini N, Gherardi G, et al. Ameliorating hypertension and insulin resistance in subjects at increased cardiovascular risk: effects of acetyl-L-carnitine therapy. *Hypertension (Dallas, Tex : 1979)*. 2009;54(3):567-74.
21. Chegary M, Brinke H, Ruiter JP, Wijburg FA, Stoll MS, Minkler PE, et al. Mitochondrial long chain fatty acid beta-oxidation in man and mouse. *Biochimica et biophysica acta*. 2009;1791(8):806-15.
22. van Vlies N, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, et al. Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. *The Biochemical journal*. 2005;387(Pt 1):185-93.
23. Ventura FV, Costa CG, Struys EA, Ruiter J, Allers P, Ijlst L, et al. Quantitative acylcarnitine profiling in fibroblasts using [U-13C] palmitic acid: an improved tool for the diagnosis of

- fatty acid oxidation defects. Clinica chimica acta; international journal of clinical chemistry. 1999;281(1-2):1-17.
24. Lindeboom L, Nabuurs CI, Hoeks J, Brouwers B, Phielix E, Kooi ME, et al. Long-echo time MR spectroscopy for skeletal muscle acetylcarnitine detection. The Journal of clinical investigation. 2014;124(11):4915-25.

Chapter 2

Isocaloric high-fat feeding decreases metabolic flexibility without alterations in insulin sensitivity and acylcarnitines species

Yvonne M.H. Bruls
Noud A. van Herpen
Joachim E. Wildberger
Matthijs K. Hesselink
Patrick Schrauwen
Vera B. Schrauwen-Hinderling

ABSTRACT

Background and aim: Recent evidence indicates that low carnitine availability may be underlying the reduced metabolic flexibility, an early hallmark in the development of type 2 diabetes. We previously found that metabolic flexibility was compromised by three weeks of isocaloric high-fat feeding, but no accumulation of intramyocellular lipids or changes in insulin sensitivity occurred. The underlying mechanism for the reduced metabolic flexibility was however not clarified. Here, we investigated whether changes in carnitine availability could account for the earlier reported compromised metabolic flexibility observed after 3-weeks of isocaloric high-fat feeding.

Methods: Nineteen overweight but healthy participants were randomly allocated to a low-fat or high-fat diet. All participants followed a 3-week low-fat run-in diet [15% energy (En%) as protein, 65 En% as carbohydrate and 20 En% as fat]. After run-in, nine participants switched to a high-fat diet [15% energy (En%) as protein, 30 En% as carbohydrate and 55 En% as fat] for three weeks whereas the other ten participants remained on the low-fat diet for 3-weeks. A hyperinsulinemic-euglycemic clamp (40 mU/m²/min), combined with indirect calorimetry (ventilated hood) was performed after 3 and 6 weeks to determine insulin sensitivity and metabolic flexibility. Free carnitine, acetylcarnitine and other acylcarnitine species were measured via tandem-mass spectrometry in plasma and skeletal muscle biopsies.

Results: Metabolic flexibility was reduced upon 3-weeks on high-fat diet compared to the low-fat diet (change in respiratory exchange ratio (Δ RER) between day 42-21: 0.02 ± 0.02 and -0.05 ± 0.01 respectively, $p=0.01$). Insulin stimulated suppression in whole-body lipid oxidation was significantly compromised ($p=0.007$) and insulin stimulation of carbohydrate oxidation was blunted after the high-fat diet compared to low-fat diet ($p=0.011$). Although metabolic flexibility was compromised, no changes in insulin sensitivity were found ($p=0.930$). Plasma and skeletal muscle free carnitine, acetylcarnitine and other acylcarnitine species were not different between the high and low-fat diets ($p>0.05$). Interestingly, free carnitine availability after run-in correlated positively with the diet induced changes in insulin sensitivity ($r=0.954$, $p<0.05$).

Conclusion: The compromised metabolic flexibility upon 3-weeks on a isocaloric high-fat diet was not accompanied by changes in plasma and skeletal muscle free carnitine availability, acetylcarnitine or other acylcarnitine species.

INTRODUCTION

Type 2 diabetes mellitus is characterized by the development of metabolic inflexibility and insulin resistance. Metabolic inflexibility is defined as the inability to switch from predominantly fat oxidation in the fasted state to carbohydrate oxidation in the insulin stimulated state (1). As a consequence of this metabolic inflexibility, postprandial glucose clearance is delayed and thereby leads to disturbances in glucose homeostasis. Obesity and excessive availability of lipid substrates are strongly related to insulin resistance and metabolic inflexibility (2).

Increasing lipid availability by infusion of lipid was consistently shown to cause metabolic inflexibility and insulin resistance (3, 4). Less clear is whether more physiological interventions, such as the consumption of a high fat diet lead to insulin resistance and metabolic inflexibility in humans. Studies in rodents indeed clearly indicate that also dietary manipulations which change fat availability can influence both metabolic flexibility and insulin sensitivity (5-8). Ad libitum high fat feeding was shown to increase plasma free fatty acid availability and to cause insulin resistance and very pronounced metabolic inflexibility which was evidenced by unresponsiveness of glucose and fatty acid oxidation rates post-prandially compared to fasted oxidation rates (5-8). In humans, consumption of a high-fat diet is also suggested to cause insulin resistance and metabolic inflexibility, however, evidence is very limited. Human studies have shown that upon consumption of a high fat diet, increased intramyocellular lipids (IMCL) (9, 10), elevated intrahepatic lipid content (IHL) (11) and enhanced β -oxidation intermediates, such as long chain acyl-CoA and long chain acylcarnitine species (5, 12) occur, and these are all linked to the development of insulin resistance and metabolic inflexibility. However, few of these studies have directly measured insulin sensitivity. Moreover, in most studies applying a high-fat diet in humans, also energy intake is increased and therefore the effect of high-fat feeding per se versus increased energy intake is difficult to dissociate. To this end, isocaloric studies of high-fat feeding are needed and therefore, we investigated previously the effects of isocaloric high-fat diet on skeletal muscle lipid accumulation, metabolic flexibility and insulin sensitivity in humans (13). We found that metabolic flexibility was compromised by three weeks of isocaloric high-fat feeding, but no accumulation of intramyocellular lipids or changes in insulin sensitivity occurred. The underlying mechanism for the reduced metabolic flexibility was however not clarified.

Recently, it has been postulated that changes in carnitine availability might play an important role in the development of metabolic inflexibility and insulin resistance (12, 14-16). Excessive substrate- and lipid availability may oversupply fat oxidative pathways, resulting in accumulation of long-chain acylcarnitines as intermediates of fat oxidation. This amassment of long-chain acylcarnitines will thereby lead to trapping of carnitine, reducing the availability of free carnitine. Indeed, high-fat feeding in rats decreased carnitine availability (15, 17) and concomitantly compromised metabolic flexibility and insulin sensitivity in these rats.

It is not yet clear how carnitine availability is linked to metabolic flexibility and insulin sensitivity, but the role that carnitine can play in converting surplus of acetyl-CoA to acetylcarnitine may be important in this. During high carbon load, acetyl-CoA production exceeds acetyl-CoA use by the tricarboxylic acid (TCA) cycle, which may lead to intra-mitochondrial accumulation of acetyl-CoA. These elevated intra-mitochondrial acetyl-CoA levels are known to inhibit pyruvate dehydrogenase (PDH) activity, and thereby lead to reduced glucose oxidation and hampered metabolic flexibility. The formation of acetylcarnitine from acetyl-CoA may serve as a mitochondrial rescue mechanism to maintain low intra-mitochondrial acetyl-CoA levels and thereby relieves PDH inhibition (14-16). As a result, metabolic flexibility would be maintained. However, this process depends on the availability of free carnitine and is catalyzed by the enzyme carnitine acyltransferase (CrAT). Indeed, a mouse model in which CrAT protein was knocked out was characterized by severely hampered metabolic flexibility. Furthermore, in diabetic mice (BAP-agouti transgenic mice) that are characterized by decreased metabolic flexibility also reduced skeletal muscle acetylcarnitine concentrations were reported and elevating free carnitine availability in these mice improved acetylcarnitine concentrations as well as metabolic flexibility and insulin sensitivity (18).

Therefore, low free carnitine availability may be a limiting factor in the proposed mitochondrial rescue mechanism by hampering acetylcarnitine formation, leading to a reduction in metabolic flexibility. Based on these results, it is tempting to speculate that low free carnitine availability might underlie the reduction in metabolic flexibility that is observed upon high-fat feeding. Here, we investigated whether changes in carnitine availability could account for the earlier reported compromised metabolic flexibility observed after 3-weeks of isocaloric high-fat feeding.

METHODS

Subjects

Nineteen sedentary, healthy overweight men (age: 40-70 years, BMI: 25-30 kg/m²) participated in this study. Participants had stable body weight (weight gain or loss < 3 kg in the previous 3 months) and no type 2 diabetes mellitus. The study protocol was approved by the institutional Medical Ethical Committee and was performed in accordance with the declaration of Helsinki. All participants gave written informed consent after explanation of the study protocol and potential risks. In the current study, the role of acylcarnitine species upon isocaloric high fat feeding was investigated in relation to insulin sensitivity and metabolic flexibility. Data regarding insulin sensitivity and metabolic flexibility were already published by van Herpen et al. 2011 (13).

Experimental design

This study was performed in a parallel design. After inclusion, participants were subjected to a run-in period of 3 weeks during which participants consumed a low-fat diet. At the end of this run-in period (day 21), baseline measurements were conducted. A hyperinsulinemic-euglycemic clamp was performed to assess peripheral insulin sensitivity. Before and at the end of the hyperinsulinemic-euglycemic clamp, muscle biopsies were taken from the vastus lateralis muscle to determine skeletal muscle acylcarnitine species. Subsequently, participants were randomly assigned to either a high-fat diet or continuation of the low-fat diet (control group) for 3 weeks. After 3 weeks on the high or low-fat diet (day 42), measurement of peripheral insulin sensitivity and skeletal muscle acylcarnitine species were repeated (figure 1).

Dietary intervention

All participants followed an isocaloric diet with either low or high fat content. The low-fat diet consisted of 15% energy from protein, 20% energy from fat and 65% energy from carbohydrates. In the high fat diet, protein content remained constant (15% energy) but fat content was increased (55% energy) at the expense of carbohydrate (30% energy). A detailed description of the dietary intervention is provided in van Herpen et al (13).

Hyperinsulinemic-euglycemic clamp

On day 21 and 42, all participants underwent a 3-hour hyperinsulinemic-euglycemic

Figure 1. Flowchart of the study design. Participants followed a 3-week low-fat run-in diet followed by baseline measurements of insulin sensitivity, metabolic flexibility and skeletal muscle acylcarnitines species on day 21. Subsequently, participants were randomly assigned to 3-weeks low-fat (control group) or high-fat diet. After three weeks on a low or high-fat diet, insulin sensitivity, metabolic flexibility and skeletal muscle acylcarnitine species were determined again on day 42.

clamp ($40 \text{ mU/m}^2 \cdot \text{min}$) with glucose tracer ($\text{D-[6,6-}^2\text{H}_2\text{]-glucose}$) as previously described (19). Participants reported to the university in the morning after an overnight fast. A fasted blood sample was obtained, after which a primed-continuous infusion of $\text{D-[6,6-}^2\text{H}_2\text{]-glucose}$ ($0.04 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) was started to determine rates of glucose appearance (R_a) and glucose disappearance (R_d) (20). Subsequently, a muscle biopsy was taken from the vastus lateralis muscle. After 3 hours ($t=180$), a constant infusion of insulin ($40 \text{ mU/m}^2 \cdot \text{min}$) was started combined with simultaneous infusion of glucose (glucose 20%) to maintain euglycemia (5.0 mmol/L). During the basal period ($t=150-180$) and under steady state clamp conditions ($t=270-300$), blood samples were collected and indirect calorimetry (ventilated hood) was performed to calculate substrate oxidation according to Frayn (21) and determine metabolic flexibility. At the end of the insulin stimulated steady state ($t=300$), a second muscle biopsy was taken from the vastus lateralis muscle.

Tracer calculations

Isotopic enrichment of plasma glucose was determined by electron ionization gas chromatography mass spectrometry as described previously (22). Steeles single pool non-steady state equations were used to calculate glucose R_a and R_d (20). Volume of distribution was assumed to be 0.160 L/kg for glucose. Insulin-stimulated glucose disposal (ΔR_d) was computed as the difference between R_d under insulin-stimulated conditions and R_d under basal non-insulin stimulated conditions. EGP was calculated as R_a minus exogenous glucose infusion rate. Non-oxidative glucose disposal (NOGD) was

calculated as R_d minus carbohydrate oxidation, derived from the ventilated hood measurements.

Muscle biopsies

On day 21 and 42, muscle biopsies were taken from the vastus lateralis muscle under local anesthesia (2% Lidocaine), according to the Bergström technique (23). The muscle tissue was directly frozen in melting isopentane and stored at -80°C until further processed. Skeletal muscle acylcarnitines were analyzed as previously described using tandem mass spectrometry (24). Total short-chain acylcarnitine species include the sum of C3-C4-C5:1-C5-C4-3OH-C4DC carnitine species. C6 until C12 acylcarnitine species summed are defined as medium-chain acylcarnitines. Total long-chain acylcarnitine species represent the sum of C14 until C18-carnitine species.

Statistics

All data are reported as mean $\pm$ SEM. SPSS 24.0 software (SPSS, Chicago, IL) was used to perform statistical analysis. To test for differences in the response to the two diets (low-fat and high-fat), students independent sample t-test were performed comparing the changes of each group from the run-in and experimental periods (deltas between day 21 and day 42). Data were considered significantly different when P value < 0.05 (two-sided testing). Using individual data, Pearson correlation coefficients were calculated to test for associations between parameters.

RESULTS

General characteristics

19 overweight participants were included, of which 10 were assigned to the low-fat diet group and nine participants were assigned to the high-fat diet group. BMI, age, body composition and maximal oxygen uptake ($\text{VO}_{2\text{max}}$) were similar between groups. Fasting glucose, insulin, free fatty acid (FFA) levels and blood lipid profiles were comparable between groups ($p > 0.05$, table 1).

Table 1. Baseline participant characteristics

	Low-fat group (N=10)	High-fat group (N=10)
Age (y)	54.0 $\pm$ 2.3	56.4 $\pm$ 2.5
BMI (kg/m^2)	29.3 $\pm$ 0.6	28.3 $\pm$ 0.5
Body weight (kg)	92.0 $\pm$ 2.9	91.3 $\pm$ 1.8
Body composition		
Fat free mass (kg)	64.0 $\pm$ 1.7	65.9 $\pm$ 1.9
Fat percentage (%)	30.1 $\pm$ 1.3	27.5 $\pm$ 1.7
Physical fitness		
$\text{VO}_{2\text{max}}$ ($\text{ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$)	33.6 $\pm$ 1.9	33.5 $\pm$ 1.7
Plasma metabolites		
Fasting glucose (mmol/L)	5.7 $\pm$ 0.1	5.7 $\pm$ 0.1
Fasting insulin (pmol/L)	15.7 $\pm$ 2.2	16.7 $\pm$ 2.8
Plasma FFA ($\mu\text{mol}/\text{L}$)	301 $\pm$ 30	416 $\pm$ 59
Blood lipid profile		
Total cholesterol (mmol/L)	5.73 $\pm$ 0.38	5.51 $\pm$ 0.39
HDL cholesterol (mmol/L)	1.23 $\pm$ 0.08	1.25 $\pm$ 0.06
LDL cholesterol (mmol/L)	4.01 $\pm$ 0.35	3.96 $\pm$ 0.35
Triglycerides (mmol/L)	1.72 $\pm$ 0.43	1.48 $\pm$ 0.16

Data are represented as mean $\pm$ SEM. FFA = Free Fatty Acids, TG = Triglyceride, LDL = Low-Density Lipoprotein and HDL = High-Density Lipoprotein

Metabolic flexibility

As previously reported (13), metabolic flexibility expressed as the change in respiratory exchange ratio upon insulin stimulation (ΔRER), was comparable after run-in ($p>0.05$) but significantly reduces upon high-fat feeding (table 2, $p=0.009$). Basal whole-body lipid and carbohydrate oxidation were similar after run-in and not affected by the dietary intervention (table 2). Insulin stimulation suppressed whole-body lipid oxidation and stimulated carbohydrate oxidation to a similar extend after run-in in the two groups. However, insulin stimulated suppression in whole-body lipid oxidation was significantly compromised ($p=0.007$) and insulin stimulation of carbohydrate oxidation was blunted after the high-fat diet compared to low-fat diet ($p=0.011$).

Insulin sensitivity

To examine whether high-fat feeding compromises peripheral insulin sensitivity, a hyperinsulinemic euglycemic clamp was performed combined with tracers to allow the determination of the rate of disappearance of glucose, which is mainly reflected by skeletal muscle glucose uptake. As expected, upon run-in the insulin stimulated rate of disappearance of glucose (ΔR_d) was similar in both groups. Surprisingly, the high-fat diet did not affect insulin stimulated glucose disposal ($p=0.927$, table 2).

Plasma acylcarnitine profiles

Fasting plasma acylcarnitine profiles were measured after an overnight fast after the run-in and after the diet intervention, and the dietary effect is expressed as the delta between day 21 and 42. The change in free carnitine ($p=0.35$, figure 2A) and acetylcarnitine concentrations ($p=0.39$, figure 2B) was not different upon the high-fat diet when compared to the low-fat diet. In line with this, the change in plasma short-chain acylcarnitines (figure 2C) as well as long-chain acylcarnitine (figure 2D) did not alter upon three weeks of isocaloric high-fat diet compared to the low-fat diet. The concentrations of individual acylcarnitine species are listed in supplementary table 1.

Figure 2. Response of plasma acylcarnitine concentrations to the three-week dietary intervention. The delta (day 42 - day 21) of free carnitine (A), acetylcarnitine (B), short-chain acylcarnitines (C), medium-chain acylcarnitines (D) and long-chain acylcarnitines (E) is depicted with black bars representing the LF/LF group and white bars the LF/HF group. Data are expressed as means $\pm$ SEM. No significant differences were found between groups.

Table 2. Substrate kinetics at the end of the run-in and experimental periods.

	Low-fat group		High-fat group		P-values for difference in changes
	Run-in period	Experimental period	Run-in period	High-fat group Changes	
RQ	Basal	0.82 ± 0.1	0.81 ± 0.1	0.81 ± 0.1	P=0.75
	Clamp	0.92 ± 0.1	0.92 ± 0.1	0.92 ± 0.1	P=0.01
	Delta	0.10 ± 0.1	0.11 ± 0.1	0.10 ± 0.1	P=0.01
Rd Glucose (μmol/(kg*min))	Basal	10.4 ± 0.3	10.0 ± 0.4	10.0 ± 0.4	P=0.96
	Clamp	29.1 ± 2.9	30.2 ± 1.9	30.2 ± 1.9	P=0.92
	Delta	18.7 ± 3.0	20.1 ± 2.0	20.1 ± 2.0	P=0.93
CHO oxidation (μmol/(kg*min))	Basal	8.2 ± 1.0	7.0 ± 0.8	7.0 ± 0.8	P=0.84
	Clamp	14.9 ± 1.2	14.7 ± 0.6	14.7 ± 0.6	P=0.03
	Delta	6.7 ± 0.8	7.7 ± 0.5	7.7 ± 0.5	P=0.01
NOGD (μmol/(kg*min))	Basal	2.4 ± 0.8	3.2 ± 0.8	3.2 ± 0.8	P=0.84
	Clamp	14.2 ± 2.9	15.8 ± 2.3	15.8 ± 2.3	P=0.40
	Delta	11.8 ± 3.0	12.6 ± 2.0	12.6 ± 2.0	P=0.31
Lipid oxidation (μmol/(kg*min))	Basal	0.98 ± 0.09	1.05 ± 0.1	1.05 ± 0.1	P=0.74
	Clamp	0.45 ± 0.06	0.46 ± 0.1	0.46 ± 0.1	P=0.02
	Delta	-0.53 ± 0.08	-0.60 ± 0.0	-0.60 ± 0.0	P=0.01

Values refer to 10 subjects in the low-fat group and 10 subjects in the high-fat group. However substrate oxidation measurements were not calculated in 1 subject in each group. One subject felt claustrophobic underneath the hood and the measurement was aborted and one subject could not be measured due to technical problems. Values are mean ± SEM. P-values are based on the difference in changes between the low-fat group vs. the high-fat group. Adapted from van Herpen et al.(13).

Figure 3. Response of skeletal muscle acylcarnitine concentrations to the three-week dietary intervention. The delta (day 42 – day 21) of free carnitine (A), acetylcarnitine (B), short-chain acylcarnitines (C), medium-chain acylcarnitines and long-chain acylcarnitines (D) are shown with black bars representing the low-fat group and white bars the high-fat group. Data are expressed as means $\pm$ SEM.

Skeletal muscle acylcarnitine profiles

We also assessed skeletal muscle acetylcarnitine profiles in a muscle biopsy taken after an overnight fast on day 21 and day 42. The dietary intervention did not differentially affect the change in free carnitine (C0) and acetylcarnitine concentrations ($p > 0.05$,

figure 3A and 3B) after consumption of the high-fat diet compared to a low-fat diet. No alterations in the change of short-chain acylcarnitines were present upon three weeks high-fat diet compared to the low-fat diet (figure 3C) except for a reduction in C10 acylcarnitines ($p<0.05$) and a trend towards decreased C12:1 acylcarnitines ($p=0.05$, supplementary material). The change in long-chain acylcarnitine C14:2 was reduced upon the high-fat diet ($p=0.04$, supplementary material) compared to low-fat diet but other long-chain acylcarnitine species were unaffected by the dietary intervention ($p>0.05$, figure 2D). The concentrations of individual acylcarnitine species are listed in supplementary table 2.

Correlations

To investigate whether the baseline carnitine availability or the diet-induced change in carnitine availability was related to the metabolic response elicited by the diet, baseline acylcarnitine concentrations (after run-in) and the changes in acylcarnitine concentrations were correlated to the diet-induced change in metabolic flexibility and insulin sensitivity. Skeletal muscle free carnitine availability after run-in correlated positively with the diet-induced change in the increase in the rate of glucose disappearance (change in ΔR_d $r=0.710$, $p=0.002$, figure 4A) as well as with the change in insulin stimulated glucose disappearance (change in R_d insulin-stimulated, $r=0.954$, $p<0.01$, figure 4B) on whole group level.

Figure 4. Correlations. Basal carnitine availability upon run-in related to the change in R_d (A) and basal carnitine availability related to the dietary change in insulin stimulated R_d (B).

DISCUSSION

In the current study, we show that upon three weeks of isoenergetic high-fat feeding, insulin-stimulated whole-body lipid oxidation was increased and glucose oxidation was decreased, reflecting a reduced metabolic flexibility in overweight participants. Excessive lipid availability was previously associated with a mismatch between energy flux into the respiratory system and the demand of this respiratory system (5, 15, 25). During such a mismatch, intermediates of the β -oxidation such as acylcarnitine species can accumulate inside the mitochondria. The accumulation of intramitochondrial acylcarnitines can result in trapping of carnitine, decreasing the availability of free carnitine. The low availability of free carnitine may in turn negatively affect metabolic flexibility. Carnitine is needed for the formation of acetylcarnitine, to buffer acetyl-CoA levels under conditions of high substrate availability. Therefore, abundant carnitine availability can prevent high intramitochondrial concentrations of acetyl-CoA, which would cause inhibition of pyruvate dehydrogenase (PDH) activity. As PDH is very central in enabling oxidative glucose fluxes, ample availability of carnitine can favor substrate switching from predominantly fat- to glucose oxidation (5, 12, 14, 15, 25). Here, we hypothesize that the high-fat diet-induced metabolic inflexibility would be accompanied by an increased concentration of (mainly long chain-) acylcarnitines and a decrease of free carnitine in muscle, which could mechanistically explain the findings on whole body level.

However, on the whole group level, no increase in skeletal muscle long-chain acylcarnitine concentration was found upon the high-fat diet. In line with the latter, free carnitine availability was unchanged in skeletal muscle. Similarly, also plasma long-chain acylcarnitines and free carnitine concentrations were unchanged. This may seem in contradiction to earlier studies, mainly rodent studies, reporting incomplete lipid oxidation and accumulation of incomplete lipid-derived intermediates such as acylcarnitine species in skeletal muscle upon high fat diets (5, 15, 25). In these studies, free carnitine availability in muscle was reported to be decreased upon high fat diets, indeed indicating trapping of free carnitine (5, 15, 17, 26). It should be noted that in these animal studies mainly hypercaloric diets were used, accompanied by enhanced total food intake in the high-fat group, whereas we used isocaloric diets in the current study. Also in humans, most high-fat studies were performed under hypercaloric conditions. The consumption of a hypercaloric high-fat diet consisting of 55-60 E% fat for three consecutive days decreased peripheral insulin sensitivity compared to

consumption of a low fat diet (18-23 E% fat) (9). Similarly, a three day hypercaloric high-fat diet (77 E% fat) resulted in a decreased insulin sensitivity and this effect was more pronounced than on a hypercaloric high-carbohydrate diet (80 E% carbohydrates) (27). However, no data on lipid-induced intermediates such as acylcarnitines were present in these studies, and therefore the role of high-fat diet induced increases in acylcarnitines on insulin sensitivity in humans is still lacking. The intervention in the present human study is comparable to the above mentioned animal studies with respect to fat content of the diet, ranging from 45 E% fat to 60 E% fat, however differs with respect to the achieved energy balance. To disentangle the effect of high-fat feeding from a positive energy balance, we here choose to perform our human intervention with diets fed in energy balance with a realistic macronutrient composition.

Although not many, some human studies investigated the effects of isoenergetic high-fat diets on peripheral insulin sensitivity in healthy (28-30) as well as overweight volunteers (31). No change in insulin sensitivity occurred in healthy volunteers upon three weeks on an isocaloric high-fat diet (40-45 E% fat) compared to a low-fat diet (<30 E% fat) (29). Consumption of an isocaloric diet with similar macronutrient composition for 4 weeks did also not result in altered insulin sensitivity (30). In accordance, Garg et al. (31) reported no changes in insulin sensitivity with a comparable isocaloric high-fat diet for three weeks in overweight volunteers. However, very few studies have investigated the effect of isocaloric nutritional challenges on lipid-derived intermediates such as acylcarnitines in skeletal muscle in relation to insulin resistance. Five days of isocaloric high-fat feeding (65 E% of fat) did not affect plasma and skeletal muscle free carnitine availability, acetylcarnitine and long-chain acylcarnitines concentrations in both lean and overweight humans (32, 33), in line with results in the current study.

Taken together, these results suggest that a positive energy balance, induced by hypercaloric high-fat feeding, is needed such that the lipid flux into the mitochondria exceeds the metabolic demand leading to a mismatch between -oxidation and TCA cycle. In these conditions, lipid oxidation intermediates such as acylcarnitine species accumulate causing insulin resistance. Thus, the results suggest that enhanced energy intake and not macronutrient composition per se is needed to influence insulin sensitivity, metabolic flexibility and acylcarnitine species (28, 29, 34).

Although no change in free carnitine or acylcarnitine levels was found on a whole group level, interestingly, the basal availability of free carnitine seems to be predictive of the metabolic response to the diet with an initially low carnitine status favoring a stronger decrease in metabolic flexibility and insulin resistance. This suggests that an initially high carnitine availability reflects a high capacity to cope with a dietary challenge. However, future studies are needed to further investigate if a low carnitine status is a risk factor for the development of insulin resistance.

In the current study, the isocaloric high-fat diet did not change peripheral insulin sensitivity. Peripheral insulin sensitivity, mainly insulin-stimulated glucose uptake into skeletal muscle, can be separated into insulin-stimulated glucose oxidation and non-oxidative glucose disposal (mainly glycogen storage). Here we show that our isocaloric high-fat diet resulted in a reduction in insulin stimulated glucose oxidation. This result is in accordance with a previous study in humans, where consumption of a isocaloric very high-fat diet (83 E% of fat) for eleven days resulted in reduced insulin-stimulated glucose oxidation and a tendency to an increase in non-oxidative glucose disposal (NOGD) when compared to consumption of an intermediate-fat (41 E% of fat) and low-fat diet (20 E% of fat), again without any changes in peripheral insulin sensitivity. Similarly, Chokkalingam et al. compared six days of an isocaloric high-fat diet (75 E% of fat) to a low-fat diet (35 E% of fat) and a switch from insulin stimulated glucose oxidation towards non-oxidative glucose disposal was found after the high-fat nutritional challenge, again without any change in peripheral insulin sensitivity (35). In the current study, the isocaloric high-fat diet also did not change peripheral insulin sensitivity, but insulin stimulated glucose oxidation was reduced. Although not significant, non-oxidative glucose disposal seem to be increased upon the high-fat intervention. This indeed suggests a shift from glucose oxidation towards glucose storage in the form of glycogen. Most likely, this transition from glucose oxidation towards glycogen storage upon insulin stimulation can be interpreted as a physiological adaptation to the diet. By increasing fat content of the diet a concomitant reduction in carbohydrate intake occurs when maintaining protein and calorie intake. With reduced carbohydrate intake for a prolonged period of time, it is known that glycogen stores in muscle will be decreased and these low glycogen levels may stimulate glycogen storage when glucose availability becomes high, such as is the case during a hyperinsulinemic euglycemic clamp (28).

In conclusion, we here show that consumption of an isocaloric high-fat diet for three weeks increased insulin-stimulated lipid oxidation and decreased insulin-stimulated glucose oxidation, reflecting a decreased metabolic flexibility compared to an isocaloric low-fat diet. Although metabolic flexibility was reduced, high fat diet consumption did not affect insulin sensitivity, concentration of plasma and/or skeletal muscle free carnitine availability and acylcarnitine species on a whole group level. The results suggest that not a high fat diet per se, but rather a positive energy balance may be needed to trigger the accumulation of acylcarnitines and to induce insulin resistance. However, the muscle concentration of free carnitine was related to the metabolic response with the diet and therefore, volunteers with low free carnitine availability at the beginning of the dietary intervention showed the most pronounced decrease in insulin sensitivity and metabolic flexibility suggesting free carnitine availability as a predictive marker for metabolic response to a change in diet.

REFERENCES

1. Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes*. 2000;49(5):677-83.
2. Perseghin G, Ghosh S, Gerow K, Shulman GI. Metabolic defects in lean nondiabetic offspring of NIDDM parents: a cross-sectional study. *Diabetes*. 1997;46(6):1001-9.
3. Meex RC, Phielix E, Moonen-Kornips E, Schrauwen P, Hesselink MK. Stimulation of human whole-body energy expenditure by salsa is fueled by higher lipid oxidation under fasting conditions and by higher oxidative glucose disposal under insulin-stimulated conditions. *The Journal of clinical endocrinology and metabolism*. 2011;96(5):1415-23.
4. Phielix E, Meex R, Ouwens DM, Sparks L, Hoeks J, Schaart G, et al. High oxidative capacity due to chronic exercise training attenuates lipid-induced insulin resistance. *Diabetes*. 2012;61(10):2472-8.
5. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell metabolism*. 2008;7(1):45-56.
6. van den Broek NM, Ciapaite J, De Feyter HM, Houten SM, Wanders RJ, Jeneson JA, et al. Increased mitochondrial content rescues in vivo muscle oxidative capacity in long-term high-fat-diet-fed rats. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2010;24(5):1354-64.
7. Ramos-Roman MA, Sweetman L, Valdez MJ, Parks EJ. Postprandial changes in plasma acylcarnitine concentrations as markers of fatty acid flux in overweight and obesity. *Metabolism: clinical and experimental*. 2012;61(2):202-12.
8. Shulman GI. Unraveling the cellular mechanism of insulin resistance in humans: new insights from magnetic resonance spectroscopy. *Physiology (Bethesda, Md)*. 2004;19:183-90.
9. Bachmann OP, Dahl DB, Brechtel K, Machann J, Haap M, Maier T, et al. Effects of intravenous and dietary lipid challenge on intramyocellular lipid content and the relation with insulin sensitivity in humans. *Diabetes*. 2001;50(11):2579-84.
10. Schrauwen-Hinderling VB, Kooi ME, Hesselink MK, Moonen-Kornips E, Schaart G, Mustard KJ, et al. Intramyocellular lipid content and molecular adaptations in response to a 1-week high-fat diet. *Obesity research*. 2005;13(12):2088-94.
11. Westerbacka J, Lammi K, Hakkinen AM, Rissanen A, Salminen I, Aro A, et al. Dietary fat content modifies liver fat in overweight nondiabetic subjects. *The Journal of clinical endocrinology and metabolism*. 2005;90(5):2804-9.

12. Muoio DM. Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. *Cell*. 2014;159(6):1253-62.
13. van Herpen NA, Schrauwen-Hinderling VB, Schaart G, Mensink RP, Schrauwen P. Three weeks on a high-fat diet increases intrahepatic lipid accumulation and decreases metabolic flexibility in healthy overweight men. *The Journal of clinical endocrinology and metabolism*. 2011;96(4):E691-5.
14. Muoio DM, Noland RC, Kovalik JP, Seiler SE, Davies MN, DeBalsi KL, et al. Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. *Cell metabolism*. 2012;15(5):764-77.
15. Noland RC, Koves TR, Seiler SE, Lum H, Lust RM, Ilkayeva O, et al. Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control. *The Journal of biological chemistry*. 2009;284(34):22840-52.
16. Stephens FB, Constantin-Teodosiu D, Greenhaff PL. New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *The Journal of physiology*. 2007;581(Pt 2):431-44.
17. Wessels B, van den Broek NM, Ciapaite J, Houten SM, Wanders RJ, Nicolay K, et al. Carnitine supplementation in high-fat diet-fed rats does not ameliorate lipid-induced skeletal muscle mitochondrial dysfunction in vivo. *American journal of physiology Endocrinology and metabolism*. 2015;309(7):E670-8.
18. Power RA, Hulver MW, Zhang JY, Dubois J, Marchand RM, Ilkayeva O, et al. Carnitine revisited: potential use as adjunctive treatment in diabetes. *Diabetologia*. 2007;50(4):824-32.
19. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *The American journal of physiology*. 1979;237(3):E214-23.
20. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. *Annals of the New York Academy of Sciences*. 1959;82:420-30.
21. Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. *Journal of applied physiology: respiratory, environmental and exercise physiology*. 1983;55(2):628-34.
22. Ackermans MT, Pereira Arias AM, Bisschop PH, Endert E, Sauerwein HP, Romijn JA. The quantification of gluconeogenesis in healthy men by (2)H₂O and [2-(13)C]glycerol yields different results: rates of gluconeogenesis in healthy men measured with (2)H₂O are higher than those measured with [2-(13)C]glycerol. *The Journal of clinical endocrinology and metabolism*. 2001;86(5):2220-6.

23. Bergstrom J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance. *Acta physiologica Scandinavica*. 1967;71(2):140-50.
24. van Vlies N, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, et al. Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. *The Biochemical journal*. 2005;387(Pt 1):185-93.
25. Koves TR, Li P, An J, Akimoto T, Slentz D, Ilkayeva O, et al. Peroxisome proliferator-activated receptor-gamma co-activator 1alpha-mediated metabolic remodeling of skeletal myocytes mimics exercise training and reverses lipid-induced mitochondrial inefficiency. *The Journal of biological chemistry*. 2005;280(39):33588-98.
26. Wang ZQ, Zhang XH, Yu Y, Poulev A, Ribnicky D, Floyd ZE, et al. Bioactives from bitter melon enhance insulin signaling and modulate acyl carnitine content in skeletal muscle in high-fat diet-fed mice. *The Journal of nutritional biochemistry*. 2011;22(11):1064-73.
27. Jordy AB, Serup AK, Karstoft K, Pilegaard H, Kiens B, Jeppesen J. Insulin sensitivity is independent of lipid binding protein trafficking at the plasma membrane in human skeletal muscle: effect of a 3-day, high-fat diet. *American journal of physiology Regulatory, integrative and comparative physiology*. 2014;307(9):R1136-45.
28. Bisschop PH, de Metz J, Ackermans MT, Endert E, Pijl H, Kuipers F, et al. Dietary fat content alters insulin-mediated glucose metabolism in healthy men. *The American journal of clinical nutrition*. 2001;73(3):554-9.
29. Borkman M, Campbell LV, Chisholm DJ, Storlien LH. Comparison of the effects on insulin sensitivity of high carbohydrate and high fat diets in normal subjects. *The Journal of clinical endocrinology and metabolism*. 1991;72(2):432-7.
30. Thomsen C, Rasmussen O, Christiansen C, Pedersen E, Vesterlund M, Storm H, et al. Comparison of the effects of a monounsaturated fat diet and a high carbohydrate diet on cardiovascular risk factors in first degree relatives to type-2 diabetic subjects. *European journal of clinical nutrition*. 1999;53(10):818-23.
31. Garg A, Grundy SM, Unger RH. Comparison of effects of high and low carbohydrate diets on plasma lipoproteins and insulin sensitivity in patients with mild NIDDM. *Diabetes*. 1992;41(10):1278-85.
32. Boyle KE, Canham JP, Consitt LA, Zheng D, Koves TR, Gavin TP, et al. A high-fat diet elicits differential responses in genes coordinating oxidative metabolism in skeletal muscle of lean and obese individuals. *The Journal of clinical endocrinology and metabolism*. 2011;96(3):775-81.

33. Baker PR, 2nd, Boyle KE, Koves TR, Ilkayeva OR, Muoio DM, Houmard JA, et al. Metabolomic analysis reveals altered skeletal muscle amino acid and fatty acid handling in obese humans. *Obesity* (Silver Spring, Md). 2015;23(5):981-8.
34. Riccardi G, Giacco R, Rivellese AA. Dietary fat, insulin sensitivity and the metabolic syndrome. *Clinical nutrition* (Edinburgh, Scotland). 2004;23(4):447-56.
35. Chokkalingam K, Jewell K, Norton L, Littlewood J, van Loon LJ, Mansell P, et al. High-fat/low-carbohydrate diet reduces insulin-stimulated carbohydrate oxidation but stimulates nonoxidative glucose disposal in humans: An important role for skeletal muscle pyruvate dehydrogenase kinase 4. *The Journal of clinical endocrinology and metabolism*. 2007;92(1):284-92.

SUPPLEMENTARY DATA

Supplementary Fig. 1. Response of plasma acylcarnitine concentrations to the three-week dietary intervention. The delta (day 42 – day 21) of short and medium-chain acylcarnitines (A), long-chain acylcarnitines (B) is depicted with black bars representing the low-fat group and white bars the high-fat group. Data are expressed as means $\pm$ SEM. No significant differences were found between groups.

Supplementary Fig. 2. Response of skeletal muscle acylcarnitine concentrations to the three-week dietary intervention. The delta (day 42 – day 21) of short and medium-chain acylcarnitines (A) and long-chain acylcarnitines (B) are shown with black bars representing the low-fat group and white bars the high-fat group. Data are expressed as means $\pm$ SEM. * significantly different from LF/LF group, # tending to be different from LF/LF group.

Supplementary Table 1. Plasma acylcarnitine before and after 3 weeks of low or high-fat feeding

Plasma acyl-carnitine species ($\mu\text{mol/L}$)	Low-fat group		High-fat group		Low-fat group	High-fat group	P-values for difference in changes
	Run-in period	Experimental period	Run-in period	Experimental period	Changes	Changes	
C0-carnitine (vrij)	41.92 $\pm$ 1.38	40.71 $\pm$ 2.58	40.58 $\pm$ 2.22	41.57 $\pm$ 2.10	-1.213 $\pm$ 1.762	1.723 $\pm$ 1.472	p=0.35
C2-carnitine	7.78 $\pm$ 0.39	8.99 $\pm$ 1.18	7.05 $\pm$ 0.64	7.02 $\pm$ 0.45	1.209 $\pm$ 1.342	-0.130 $\pm$ 0.626	P=0.39
C3-carnitine	0.37 $\pm$ 0.02	0.37 $\pm$ 0.02	0.42 $\pm$ 0.04	0.53 $\pm$ 0.07	-0.001 $\pm$ 0.019	0.132 $\pm$ 0.072	p=0.14
C4-carnitine + C4-iso-carnitine	0.32 $\pm$ 0.02	0.32 $\pm$ 0.03	0.30 $\pm$ 0.02	0.35 $\pm$ 0.04	-0.002 $\pm$ 0.021	0.052 $\pm$ 0.046	P=0.35
C4:1-3M-carnitine + C4:1-2M-carnitine + C5:1-carnitine	0.05 $\pm$ 0.01	0.05 $\pm$ 0.01	0.06 $\pm$ 0.01	0.06 $\pm$ 0.01	-0.001 $\pm$ 0.006	-0.002 $\pm$ 0.009	P=0.78
C5-iso-carnitine + C4-2M-carnitine	0.16 $\pm$ 0.03	0.16 $\pm$ 0.02	0.15 $\pm$ 0.02	0.20 $\pm$ 0.04	0.002 $\pm$ 0.022	0.062 $\pm$ 0.029	p=0.16
C4-3-OH-carnitine + C4-3-OH-iso-carnitine	0.11 $\pm$ 0.01	0.15 $\pm$ 0.01	0.11 $\pm$ 0.01	0.15 $\pm$ 0.02	0.040 $\pm$ 0.021	0.039 $\pm$ 0.018	p=0.97
C6-carnitine	0.08 $\pm$ 0.01	0.07 $\pm$ 0.01	0.07 $\pm$ 0.00	0.07 $\pm$ 0.00	-0.009 $\pm$ 0.007	-0.002 $\pm$ 0.003	p=0.29
C5-OH-carnitine	0.04 $\pm$ 0.00	0.04 $\pm$ 0.00	0.04 $\pm$ 0.00	0.03 $\pm$ 0.00	-0.003 $\pm$ 0.002	-0.002 $\pm$ 0.005	p=0.79

C8:1-carnitine	0.20±0.02	0.21±0.03	0.22±0.03	0.20±0.02	0.006±0.032	-0.003±0.028	p=0.66
C8-carnitine	0.11±0.01	0.10±0.01	0.10±0.01	0.11±0.01	-0.003±0.009	0.014±0.010	p=0.18
C3-DC-carnitine	0.06±0.01	0.06±0.01	0.06±0.00	0.05±0.01	0.004±0.008	-0.003±0.008	p=0.46
C10:2-carnitine	0.04±0.00	0.03±0.00	0.04±0.00	0.04±0.00	-0.004±0.004	-0.004±0.006	p=0.95
C10:1-carnitine	0.10±0.01	0.10±0.01	0.09±0.01	0.10±0.01	0.008±0.003	0.014±0.010	p=0.69
C10-carnitine	0.12±0.01	0.11±0.01	0.10±0.02	0.12±0.02	-0.006±0.013	0.012±0.010	p=0.23
C4-DC-carnitine	0.04±0.00	0.04±0.00	0.03±0.00	0.04±0.00	-0.002±0.005	0.004±0.005	p=0.44
C5-DC-carnitine	0.06±0.01	0.06±0.00	0.06±0.00	0.06±0.01	0.001±0.007	0.006±0.007	p=0.76
C5:1-3M-DC-carnitine + C12-carnitine	0.05±0.00	0.05±0.01	0.04±0.00	0.04±0.00	-0.001±0.005	0.003±0.004	p=0.64
C5-3M-DC-carnitine + C6-DC-carnitine	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.002±0.004	0.002±0.001	p=0.76
C5-3M-3OH-DC-carnitine	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.000±0.002	-0.001±0.002	p=1.00
C14:2-carnitine	0.04±0.00	0.04±0.00	0.03±0.00	0.03±0.00	-0.006±0.006	0.000±0.004	p=0.33

C14:1-carnitine	0.07±0.00	0.07±0.01	0.05±0.00	0.05±0.01	0.001±0.009	-0.002±0.006	p=0.83
C14-carnitine + C8:1-DC-carnitine	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	-0.002±0.002	0.000±0.003	p=0.42
C8-DC-carnitine	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	-0.001±0.002	0.000±0.002	p=0.66
C14-OH-carnitine	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.001±0.003	0.000±0.002	p=0.74
C16:1-carnitine	0.03±0.00	0.03±0.00	0.02±0.00	0.02±0.00	0.002±0.001	0.002±0.002	p=0.77
C16-carnitine + C10:1-DC-carnitine	0.10±0.00	0.10±0.00	0.09±0.01	0.08±0.00	0.001±0.005	-0.007±0.004	p=0.34
C10-DC-carnitine	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	-0.004±0.005	-0.006±0.002	p=0.65
C16:1-OH-carnitine	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.002±0.001	0.002±0.001	p=0.72
C16-OH-carnitine	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.000±0.000	0.002±0.001	p=0.17
C10-OH-carnitine DC	0.01±0.00	0.00±0.00	0.00±0.00	0.01±0.00	-0.003±0.002	0.001±0.002	p=0.16
C18:2-carnitine	0.05±0.00	0.05±0.00	0.04±0.00	0.04±0.00	0.001±0.004	0.003±0.003	p=0.87
C18:1-carnitine	0.11±0.00	0.12±0.01	0.09±0.01	0.09±0.01	0.011±0.007	0.003±0.008	p=0.63

C18-carnitine	0.05±0.00	0.05±0.00	0.04±0.00	0.05±0.00	-0.002±0.004	0.007±0.004	p=0.12
C18:1-OH-carnitine	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.002±0.001	0.001±0.001	p=0.49
C18-OH-carnitine	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.001±0.002	0.001±0.003	p=0.97

Values refer to 9 subjects in the low-fat group and 10 subjects in the high-fat group. Values are mean ± SEM. P-values are based on the difference in changes between the low-fat group vs. the high-fat group.

Supplementary Table. 2. Skeletal muscle acylcarnitine concentration (in biopsies) before and after 3 weeks of low or high-fat feeding

Skeletal muscle acyl-carnitine species (pmol/mgdw)	Low-fat group		High-fat group		Low-fat group	High-fat group	P-values for difference in changes
	Run-in period	Experimental period	Run-in period	Experimental period			
C0	5799.48± 1476.65	8899.96± 1309.09	4326.64± 1096.32	7728.61± 1569.39	3100.48± 1759.89	3401.97± 1886.44	p=0.91
C2	465.28± 145.97	661.69± 107.29	771.90± 326.49	818.30± 279.60	196.42± 136.14	46.41± 503.47	p=0.78
C3	8.33±2.15	12.24±3.10	8.68±1.56	15.73±6.98	3.91±2.36	7.04±6.19	p=0.64
C4	4.28±0.79	6.47±0.98	5.12±2.17	4.26±1.10	2.19±1.35	-0.86±2.59	p=0.31
C5:1	0.72±0.33	0.77±0.26	0.48±0.19	2.82±2.37	0.05±0.10	2.34±2.29	p=0.33
C5	4.01±1.21	4.19±1.65	1.46±0.36	1.78±0.49	0.18±1.20	0.32±0.70	p=0.92
C4-3OH	1.85±0.52	3.03±0.92	3.66±2.02	8.05±3.19	1.18±1.02	4.39±4.09	p=0.46
C6	1.15±0.23	1.68±0.33	1.61±0.46	1.13±0.28	0.54±0.36	-0.49±0.56	p=0.15
C8:1	0.61±0.15	0.92±0.25	0.66±0.17	1.70±0.87	0.31±0.32	1.04±0.89	p=0.45
C8	0.51±0.07	0.81±0.18	0.86±0.24	1.85±1.17	0.30±0.20	0.99±1.17	p=0.57
C4DC	0.62±0.15	0.86±0.10	0.78±0.18	1.80±1.05	0.24±0.15	1.02±1.03	p=0.46
C10	0.20±0.03	0.46±0.11	0.42±0.13	0.29±1.07	0.25±0.12	-0.13±0.13	p=0.05
C12:1	0.08±0.02	0.20±0.06	0.18±0.05	0.10±0.04	0.12±0.06	-0.08±0.07	p=0.05
C12	0.37±0.05	0.63±0.14	0.51±0.13	1.10±0.59	0.26±0.16	0.59±0.58	p=0.60
C14:2	0.13±0.03	0.32±0.07	0.30±0.09	0.18±0.06	0.19±0.08	-0.12±0.10	p=0.04
C14:1	0.36±0.08	0.78±0.30	0.73±0.26	0.40±0.16	0.42±0.35	-0.33±0.32	p=0.13
C14	0.51±0.12	0.90±0.24	0.70±0.23	0.48±0.18	0.40±0.31	-0.22±0.24	p=0.14
C16:2	0.21±0.06	0.22±0.05	0.21±0.07	0.94±0.72	0.02±0.08	0.74±0.69	p=0.31

C16:1		0.47±0.11	1.04±0.33	0.62±0.21	1.22±0.76	0.57±0.36	0.60±0.78	p=0.97
C16		3.02±0.72	4.72±1.03	3.48±0.69	2.53±0.56	1.70±1.45	-0.94±0.66	p=0.12
C12:1DC or C16:1-OH		0.13±0.03	0.19±0.04	0.17±0.05	0.15±0.05	0.06±0.06	-0.03±0.07	p=0.38
C18:2		0.98±0.25	1.60±0.45	1.38±0.45	1.91±1.00	0.63±0.55	0.53±1.14	p=0.94
C18:1		3.00±0.63	5.21±1.39	3.71±0.95	2.99±0.69	2.21±1.75	-0.72±1.06	p=0.17
C18		1.61±0.51	1.98±0.34	1.78±0.43	1.12±0.33	0.37±0.67	-0.66±0.37	p=0.20
C20:2		0.12±0.09	0.07±0.01	0.08±0.02	0.07±0.02	-0.05±0.08	-0.01±0.03	p=0.69
C20:1		0.28±0.19	0.11±0.02	0.13±0.03	1.28±1.18	-0.17±0.20	1.15±1.19	p=0.29
C20		0.21±0.17	0.04±0.01	0.08±0.02	0.05±0.01	-0.16±0.17	-0.03±0.02	p=0.44

Values refer to 8 subjects in the low-fat group and 8 subjects in the high-fat group. Values are mean ± SEM. P-values are based on the difference in changes between the low-fat group vs. the high-fat group.

Chapter 3

L-carnitine infusion does not alleviate lipid-induced insulin resistance and metabolic inflexibility

Yvonne M.H. Bruls

Yvo J.M. op den Kamp

Esther Phielix

Lucas Lindeboom

Bas Havekes

Joachim E. Wildberger

Matthijs K.C. -Hesselink

Patrick Schrauwen

Vera B. Schrauwen-Hinderling

ABSTRACT

It has been suggested that a low carnitine status may underlie the development of insulin resistance and metabolic inflexibility. Intravenous lipid infusion elevates plasma free fatty acid concentration and is a model for compromised insulin sensitivity and metabolic flexibility in healthy, insulin sensitive volunteers. We hypothesized that co-infusion of L-carnitine alleviates lipid-induced insulin resistance and metabolic inflexibility. In a crossover design, eight young healthy volunteers underwent hyperinsulinemic-euglycemic clamps ($40 \text{ mU/m}^2/\text{min}$) with simultaneous infusion of saline (control), Intralipid (20%, 90 ml/h) (LIPID), or Intralipid (20%, 90 ml/h) combined with infusion of L-carnitine (28 mg/kg/h) (LIPID+CAR). Plasma free carnitine availability increased upon carnitine infusion (from 34.4 ± 1.9 to $183.3 \pm 6.3 \text{ } \mu\text{mol/L}$, $p < 0.01$). Insulin stimulation resulted in suppression of plasma free fatty acids (FFA) compared to baseline in CON (32.2 ± 1.9 and $389.0 \pm 46.7 \text{ } \mu\text{mol/L}$ respectively, $p < 0.01$). Additional lipid infusion with insulin increased plasma FFA levels compared to the control condition (1034.9 ± 107.3 $p < 0.01$). The FFA levels increased to a similar extent in LIPID+CAR compared to LIPID ($1028.7 \pm 99.6 \text{ } \mu\text{mol/L}$, $p > 0.05$). Inherent to the lipid infusion model, peripheral insulin sensitivity was blunted upon lipid infusion compared to the control condition (M-value: 26.0 ± 3.1 and $52.5 \pm 3.8 \text{ } \mu\text{mol/kg/min}$ respectively, $p < 0.01$). L-carnitine infusion did not alleviate lipid-induced insulin resistance (M-value: $25.3 \pm 4.0 \text{ } \mu\text{mol/kg/min}$, $p > 0.99$). Metabolic flexibility decreased upon lipid infusion compared to control (ΔRER 0.01 ± 0.01 and 0.10 ± 0.02 , $p < 0.01$) but was not alleviated by L-carnitine infusion (0.01 ± 0.01 , $p > 0.05$). Upon 6 hours of lipid infusion lipid oxidation increased ($p < 0.01$) with a concomitant decrease in glucose oxidation ($p < 0.01$). These observations were not affected by infusion of L-carnitine ($p > 0.05$). No difference in skeletal muscle free carnitine or acetylcarnitine were found between CON, LIPID and LIPID+CAR ($P > 0.05$). Insulin stimulation reduced short and medium-chain acylcarnitines. This suppression tended to be blunted in LIPID and LIPID+CAR, without difference between LIPID and LIPID+CAR. Long-chain acylcarnitines tended to be decreased upon insulin stimulation. This suppression remained unaffected in LIPID and LIPID+CAR, although a tendency to decreased long-chain acylcarnitine in LIPID compared to CAR ($p < 0.10$). In conclusion, we show that insulin resistance and metabolic inflexibility induced by lipid infusion could not be alleviated by acute carnitine infusion possibly due to the lack of carnitine uptake in the skeletal muscle

INTRODUCTION

Type 2 diabetes mellitus is an increasing health problem worldwide. Type 2 diabetes patients and individuals at risk of developing diabetes are characterized by insulin resistance and metabolic inflexibility (1). The latter is defined as an impaired capacity to switch from lipid oxidation in the fasted state towards carbohydrate oxidation in the insulin stimulated state (1). Obesity and excessive availability of lipid substrate are strongly related to insulin resistance and metabolic inflexibility (2) and lipid infusion in insulin sensitive subjects reduces insulin sensitivity and metabolic flexibility (3, 4).

It has been recently suggested that carnitine might play a crucial role in maintaining insulin sensitivity and metabolic flexibility. Although carnitine is best known for its function in the transport of long-chain fatty acyl-CoA into the mitochondrial matrix, allowing subsequent β -oxidation (5, 6), it also has other functions. The function of carnitine to conjugate with acetyl-CoA to form acetylcarnitine, facilitated by the enzyme carnitine acyl transferase (CrAT) in the mitochondria is gaining more and more interest as it may be relevant in preserving insulin sensitivity and metabolic flexibility. With reduced acetylcarnitine formation, acetyl-CoA levels start accumulating inside the mitochondria especially during conditions of high substrate load (i.e. exercise or high-fat feeding). As a consequence, pyruvate dehydrogenase (PDH) activity is inhibited leading to a mitochondrial inability to switch to glucose oxidation, so called metabolic inflexibility. The availability of carnitine is therefore crucial in acetylcarnitine formation and may be instrumental to maintain metabolic flexibility, insulin sensitivity, and glucose homeostasis (7-9).

Indeed, animal studies correlated metabolic inflexibility to changes in carnitine status (7, 8, 10). Thus, it was shown that a reduction in free carnitine availability in rats was associated with decreased metabolic flexibility and that the consumption of a high-fat diet lowered free carnitine availability. Interestingly, increasing carnitine availability via carnitine supplementation resulted in complete restoration of metabolic flexibility, PDH activity, and insulin sensitivity (8). Furthermore, decreased acetylcarnitine concentrations were shown in obese mice with insulin resistance. Again, improving carnitine availability in these mice restored acetylcarnitine levels and concomitantly improved metabolic flexibility, PDH activity, insulin sensitivity, and blood glucose levels (10).

In humans, lower skeletal muscle acetylcarnitine concentrations measured via magnetic resonance spectroscopy are reported in type 2 diabetes patients, possibly indicating a decreased carnitine availability (11). Carnitine supplementation has been shown to improve glucose tolerance in insulin resistant subjects with low carnitine status (12). Some, although not all studies, report beneficial effects of carnitine administration on plasma glucose, insulin, and lactate levels (13-17). Furthermore, markers of insulin resistance such as glucose infusion rate (GIR) (16, 17) and M-value (18) were reported to improve upon intravenous carnitine administration. However, it still remains elusive what is underlying these beneficial effects of carnitine administration.

We hypothesize that free carnitine availability in skeletal muscle tissue might be crucial in maintaining metabolic flexibility and insulin sensitivity. Especially when lipid availability is increased, free carnitine availability might become limiting. Therefore, in the current study we aimed to investigate if carnitine infusion during simultaneous lipid infusion could alleviate lipid-induced insulin resistance and metabolic inflexibility in healthy young males.

METHODS

General characteristics

Eight young, healthy sedentary lean males participated in this study. Participants were excluded in case of medication use interfering with glucose homeostasis, exercise engagement exceeding 3 hours a week, unstable body weight (weight gain or loss > 3kg in the previous 3 months), MRI contra-indications, impairments in kidney and/or renal function, uncontrolled hypertension, and cardiovascular disease. All subjects gave written informed consent after the protocol was fully explained. Study procedures were approved by the institutional Medical Ethical Committee 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 NTC02722902.

Experimental design

The study was set up as a single blind, placebo-controlled randomized cross-over design. All subjects reported five times to the university. Participants were instructed to maintain their usual physical activity patterns and to not change dietary behavior while participating in the study. During visit 1, participants reported to the university in the morning after an overnight fast. Body composition (fat percentage and fat free mass) was determined. Subsequently, maximal oxygen uptake ($\text{VO}_{2\text{max}}$) and maximal output were determined during an incremental cycling test on a stationary bike. On a separate day (visit 2), participants came to the university at 4.30 pm. After consumption of a light lunch at 12:00 am, participants remained fasted until 5:00 pm. Skeletal muscle acetylcarnitine concentrations were measured at 5:00 pm *in vivo* in the *m. vastus lateralis* using long-TE ^1H -MRS at rest, followed by a 30-minute cycling exercise at 70% of the participants predetermined maximal output, after which acetylcarnitine was reassessed. On each of the following visits (visit 3, 4 and 5), a hyperinsulinemic euglycemic clamp was performed to assess peripheral insulin sensitivity. Two hyperinsulinemic euglycemic clamps were performed with simultaneous infusion of lipids. In one of these lipid infusion trials, subjects received simultaneously L-carnitine infusion (=LIPID + CAR). In the other lipid trial, L-carnitine infusion was replaced by saline infusion (=LIPID). During the third hyperinsulinemic euglycemic clamp, only saline was infused as a control for the lipid infusion (=CON). The sequence of these different hyperinsulinemic-euglycemic clamp conditions was randomly assigned. A wash-out period of at least two weeks was used to prevent carry over effects of the carnitine and lipid infusion. Participants were blinded for the treatment.

Body composition

During the first visit, participants reported to the university after an overnight fast. Body mass and body volume were assessed using air-displacement plethysmography (ADP) using the Bod Pod device (Cosmed, Italy, Rome) according to the manufacturers instructions and as described previously (19, 20). Thoracic gas volume was predicted based on equations included in the Bod Pod software. From these data, body composition (fat mass, fat free mass and fat percentage) was calculated as described by Siri (21).

$\text{VO}_{2\text{max}}$

Directly after the body composition determination during the first visit, all participants performed a routine incremental exhaustive cycling test on a stationary bike to determine maximal oxygen uptake ($\text{VO}_{2\text{max}}$) and maximal output (W_{max}) for characterization of the participants as reported previously (22). Briefly, after a five minute warming-up period, the workload was increased every 2.5 minutes until exhaustion was reached. Oxygen uptake was measured continuously throughout the test using indirect calorimetry (Omnicol, Maastricht, The Netherlands).

Skeletal muscle acetylcarnitine formation upon exercise

During the second visit, resting skeletal muscle acetylcarnitine concentrations were measured as well as the capacity to form acetylcarnitine upon exercise. Participants were instructed to refrain from physical activity three days prior to the acetylcarnitine measurement and reported to the university at 4.30 pm after a 4.5 hour fast (since 12:00 am). Subsequently, participants rested for 30 minutes and at 5:00 pm, resting skeletal muscle acetylcarnitine concentrations were determined *in vivo* using non-invasive long echo time (TE) proton magnetic resonance spectroscopy (^1H -MRS) as previously reported (11). ^1H -MRS measurements were performed on a 3T clinical MR scanner (Achieva, 3T-X, Philips Healthcare, Best, The Netherlands). Participants were placed in supine position in the MR scanner with a two-element flex surface coil positioned around the upper leg. The volume of interest (40 mm x 20 mm x 60 mm) was positioned in the *m. vastus lateralis*. The spectra were acquired with the following acquisition parameters: PRESS sequence, TR=6000 ms, spectral bandwidth 2 kHz, number of acquired data points 2048, TE=350 ms, NSA=20. Following the resting acetylcarnitine determination, participants performed a 30-minute cycling exercise on a stationary bike at 70% of their predetermined maximal output. Immediately after the cycling exercise, skeletal muscle

acetylcarnitine formation was measured again. Baseline correction was performed for all acquired spectra with a custom-made MATLAB script (The Mathworks Inc.). Spectra were analyzed using the AMARES algorithm in jMRUI software (23). The creatine resonance (t-Cr) was used as internal reference and acetylcarnitine concentration was calculated assuming a creatine concentration of 30 mmol/kgww. T_2 corrections were performed for creatine ($T_2=166$ ms) and acetylcarnitine ($T_2=262$ ms) and a correction for the dipolar coupling of creatine was applied and set at 30% of the signal as reported earlier (11).

Hyperinsulinemic-Euglycemic clamp

At visit 3, 4 and 5, insulin sensitivity was assessed during a 6-hour hyperinsulinemic-euglycemic clamp. Participants refrained from strenuous exercise three days preceding the clamp and monitored their food intake in a food diary. A carbohydrate rich meal was consumed by all participants the evening prior to the clamp. At the day of the clamp, participants reported to university at 6.30 am after an overnight fast from 8:00 pm onwards. A fasted blood sample was obtained and a primed constant 6-hour insulin infusion was started (40 mU/m^2) with simultaneous infusion of variable amounts of glucose (glucose 20%) to maintain euglycemia (5.0 mmol/L). Next to the infusion of insulin and glucose, infusion of Intralipid or saline and L-carnitine or saline were started. Intralipid (Fresenius Kabi, Zeist, Nederland) or saline (Braun) was administrated at an infusion rate of 90 ml/h . A primed (4 mg/kg) continuous (4 mg/kg/h) infusion of L-Carnitine (Carnitene, Sigma tau, Rome, Italy) or saline (Braun, Melsungen, Germany) was used. Indirect calorimetry (ventilated hood) was performed and blood samples were taken in the basal state ($t=-30-0 \text{ min}$) and the last 30 minutes ($t=330-360 \text{ min}$) of insulin stimulation to determine metabolic flexibility and glucose and lipid oxidation rates according to Peronnet et al. (24)

Muscle biopsies

On the day of the hyperinsulinemic-euglycemic clamp (visit 3, 4 and 5), skeletal muscle biopsies were taken in the morning after an overnight fast and upon 6 hours of insulin stimulation. Muscles biopsies were taken from the *m. vastus lateralis* muscle according to the Bergstrom method (25) under local anesthesia (1% Lidocaine, Accord Healthcare Limited, Harrow, United Kingdom). Muscle tissue was immediately frozen in melting isopentane and stored at -80°C until further processing. Skeletal muscle acylcarnitine species were determined using mass spectrometry as described previously (26).

Blood sample analysis

During the hyperinsulinemic-euglycemic clamp the hand was heated in a hot box (55°C) to allow arterialized venous blood sampling from the hand vein. The arterialized venous blood samples were immediately centrifuged and plasma was frozen in liquid nitrogen and stored at -80°C until analyzed. Plasma free fatty acid (FFA) concentration were determined at t=120, 180, 240, 360 and 480 via an enzymatic assay automated on a Cobas Dara/Mira analyzer (Wako Nefa C test kit, Wako Chemicals). Plasma acylcarnitine species were measured using tandem mass spectrometry as previously described (27) during the basal and insulin stimulated steady state (t=120 and t=480) and at t=180 to determine the expected increase in plasma acetylcarnitine levels.

Statistics

The statistical analysis was performed using SPSS 24.0 software (SPSS, Chicago, IL.). All results are presented as mean $\pm$ SEM. Statistical significance was set at $P < 0.05$. A one-way ANOVA was carried out to investigate differences in insulin sensitivity (M-value), metabolic flexibility (Δ RER) and skeletal muscle acylcarnitine species after 6 hours of insulin infusion between trials. For the comparisons of skeletal muscle acylcarnitine species in the insulin-stimulated states with the basal state, a Students paired sample t-test was performed with Bonferroni correction for multiple testing and therefore a p-value of 0.0125 was considered statistically significant. A two-way ANOVA for repeated measures was performed to test differences in GIR, oxidation rates, and plasma acylcarnitines. In case of a significant F-value, Bonferroni post-hoc analyses were performed.

RESULTS

Participant characteristics

Eight healthy young lean male participants (body weight= 76.5 ± 1.9 kg, BMI= 23.2 ± 0.4 kg/m², age= 22 ± 1 year) were included. All participants were untrained and their maximal oxygen uptake (VO_{2max}) (42.4 ± 2.5 ml/min/kg) as well as the body fat percentage ($17.1 \pm 1.9\%$) were within the normal range for young, untrained males. All participant characteristics are presented in table 1. To investigate if the capacity to form acetylcarnitine could predict lipid induced insulin resistance, we assessed the change *in vivo* acetylcarnitine formation capacity upon exercise. Exercise is known to elevate skeletal muscle acetylcarnitine levels, assumingly because substrate load into the mitochondria increases rapidly with exercise causing acetyl-CoA levels to rise. Skeletal muscle acetylcarnitine concentrations were therefore determined *in vivo* via ¹H-MRS in the vastus lateralis muscle in the resting state and immediately after exercise at 70% W_{max} . Acetylcarnitine concentrations were more than doubled after exercise from 2.2 ± 0.2 mmol/kgww in the resting state to 4.5 ± 0.5 mmol/kgww upon exercise ($p < 0.05$, figure 1). No correlations were found between the capacity to form acetylcarnitine upon exercise, and insulin sensitivity or metabolic flexibility ($p > 0.05$), which may be due to the limited intra-individual variation in response to exercise.

Figure 1. Skeletal muscle acetylcarnitine concentrations in the resting state and the capacity to form acetylcarnitine with exercise. Data are expressed per individual (n=7).

Table 1. Substrate kinetics and insulin sensitivity

	N=8
Age (y)	22 ± 1
Body mass (kg)	76.5 ± 1.9
BMI (kg/m ²)	23.2 ± 0.4
Waist-Hip ratio	0.84 ± 0.02
Blood pressure	
Systolic (mmHg)	118 ± 2
Diastolic (mmHg)	72 ± 1
Body composition	
Fat mass (kg)	12.9 ± 1.6
Fat free mass (kg)	61.3 ± 2.1
Fat percentage (%)	17.1 ± 1.9
Physical fitness	
VO ₂ max (ml*min ⁻¹ *kg bw ⁻¹)	42.4 ± 2.5
Wmax (W*kgbw ⁻¹)	3.3 ± 0.2
Oral glucose tolerance test (OGTT)	
Fasting glucose (mmol/L)	4.9 ± 0.1
Fasting insulin (pmol/L)	28.9 ± 3.9
HbA1c (%)	5.2 ± 0.1
Blood lipid profile	
Total cholesterol (mmol/L)	4.1 ± 0.2
HDL cholesterol (mmol/L)	1.5 ± 0.1
LDL cholesterol (mmol/L)	2.2 ± 0.1
Triglycerides (mmol/L)	1.0 ± 0.1

Data are represented as mean ± SEM. W_{max}, maximal workload, VO_{2max} is normalized to body weight in kg

Insulin sensitivity

At baseline, plasma FFA levels were comparable between the trials (389.0 ± 46.7 , 414.8 ± 40.0 , 382.0 ± 59.5 in CON, LIPID and LIPID+CAR respectively, $p > 0.05$). In the lipid trial an increase in FFA levels occurred over time and FFA levels were significantly higher at all time points compared to the control condition ($p < 0.01$, figure 2A). Additional infusion of carnitine did not alter FFA levels when compared to lipid infusion alone ($p > 0.05$, figure 2A).

During the first three hours of the clamp, glucose infusion rates (GIR) were comparable between all three trials. From 3 hours onwards, glucose infusion rates were lower in LIPID as well as in LIPID+CAR compared to CON ($p < 0.05$), consistent with previous studies showing that lipid-induced insulin resistance occurs after 2-3 hours of lipid infusion (4, 28). No difference was found in GIR at any time point between LIPID and LIPID+CAR ($p > 0.05$) indicating that carnitine infusion did not alter lipid-induced insulin resistance (figure 2B). As a result, peripheral insulin sensitivity, expressed as the M-value, was blunted ($p < 0.01$) with lipid infusion compared to the control condition (26.0 ± 3.1 and $52.5 \pm 3.8 \mu\text{mol/kg/min}$, respectively) but the lipid-induced insulin resistance was not alleviated by carnitine infusion (M-value LIPID+CAR; $25.3 \pm 4.0 \mu\text{mol/kg/min}$, $p > 0.99$ compared to LIPID, figure 2C).

Figure 2. Plasma FFA concentrations (A), glucose infusion rates (B) and insulin sensitivity expressed as M-value in CON, LIPID and LIPID+CAR during the hyperinsulinemic-euglycemic clamp. Data are expressed as mean±SEM. *, Significantly different from CON ($P < 0.05$).

Metabolic flexibility

Basal glucose and lipid oxidation were comparable between trials (table 2). Glucose oxidation was increased by 6 hours of insulin infusion in the control condition (from 5.39 ± 1.78 to $19.6 \pm 1.5 \mu\text{mol/kg/min}$, $p < 0.01$, figure 3A), however this response was blunted by the infusion of lipids (from 6.49 ± 1.46 to $8.5 \pm 1.6 \mu\text{mol/kg/min}$, $p > 0.05$, figure 3A). Additional carnitine infusion did not change the lipid-induced suppression in glucose oxidation ($p > 0.05$). In line with these findings, lipid oxidation was elevated after 6-hours of lipid infusion compared to controls (1.8 ± 2.7 and $0.6 \pm 0.2 \mu\text{mol/kg/min}$ in LIPID and CON respectively, $p < 0.01$, figure 3B). Again, carnitine infusion did not further affect lipid oxidation rates ($p > 0.05$, table 2). No difference in basal RER were detected ($p > 0.05$, figure 3C). Metabolic flexibility, expressed as $\Delta\text{RER}_{\text{clamp-basal}}$, was decreased due to lipid infusion compared to controls (0.10 ± 0.02 and 0.01 ± 0.01 in CON and LIPID respectively, $p < 0.01$, figure 3D). Carnitine did not change the lipid-induced decrease in metabolic flexibility (0.01 ± 0.01 in LIPID+CAR, $p > 0.05$). No difference in metabolic

flexibility, glucose oxidation or lipid oxidation were found when analysis were performed in the middle of the clamp (after 2.5 hours of insulin) between LIPID and LIPID+CAR ($p>0.05$, table 2).

Table 2. Substrate kinetics and insulin sensitivity

	CON	LIPID	LIPID+CAR
RER (Arbitrary Units)			
Basal (t=-30-0)	0.78 ± 0.02	0.78 ± 0.02	0.78 ± 0.03
Middle (t=120-150)	0.90 ± 0.02	0.83 ± 0.03	0.83 ± 0.03
Insulin stimulated (t=330-360)	0.91 ± 0.02	0.78 ± 0.02 ^a	0.79 ± 0.03 ^a
$\Delta_{\text{clamp-basal}}$	0.10 ± 0.02	0.01 ± 0.01 ^a	0.01 ± 0.01 ^a
CHO oxidation ($\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)			
Basal (t=-30-0)	5.4 ± 1.8	6.5 ± 1.5	6.3 ± 2.2
Middle (t=120-150)	18.1 ± 2.3	12.6 ± 2.3	12.8 ± 3.3
Insulin stimulated (t=330-360)	19.6 ± 1.5	8.5 ± 1.6 ^a	8.5 ± 2.6 ^a
$\Delta_{\text{clamp-basal}}$	9.5 ± 2.2	1.4 ± 0.8 ^a	1.6 ± 1.2 ^a
Lipid oxidation ($\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)			
Basal (t=-30-0)	1.7 ± 0.1	1.6 ± 0.2	1.6 ± 0.2
Middle (t=120-150)	0.8 ± 0.1	1.4 ± 0.2	1.4 ± 0.3
Insulin stimulated (t=330-360)	0.6 ± 0.2	1.8 ± 0.2	1.8 ± 0.2
$\Delta_{\text{clamp-basal}}$			
M-value ($\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	52.5 ± 3.8	26.0 ± 3.1	25.3 ± 4.0
Plasma FFA ($\mu\text{mol/L}$)			
Basal (t=-30-0)	389.0 ± 46.7	414.8 ± 40.0	382.0 ± 59.5
Insulin stimulated (t=330-360)	32.2 ± 1.9	1034.9 ± 107.3	1028.7 ± 99.6

Data are expressed as mean ± SEM. ^a significantly different from CON.

Plasma acylcarnitine profiles

A time*treatment interaction was present for plasma free carnitine availability ($p<0.01$). Plasma free carnitine levels were similar at baseline (35.8 ± 2.0 , 36.3 ± 2.8 , 34.4 ± 1.9 $\mu\text{mol/L}$ in CON, LIPID, LIPID+CAR respectively, $p=0.829$, figure 4A). One hour of carnitine infusion already increased plasma free carnitine availability to supraphysiological concentrations (155.4 ± 4.9 $\mu\text{mol/L}$, $p<0.01$) and finally reaching concentrations of 183.3 ± 6.3 $\mu\text{mol/L}$ ($p<0.01$) after six hours of infusion (figure 4A). No changes from baseline in plasma free carnitine availability were observed in the CON and LIPID trial over time ($p>0.05$, figure 4A). Plasma acetylcarnitine (C2) concentrations showed a time*treatment interaction ($p<0.01$) and were comparable between trials at baseline ($p>0.861$). C2 concentrations decreased in the CON trial over time from 5.4 ± 0.7 to 3.6 ± 0.3 after one hour and to 2.3 ± 0.1 $\mu\text{mol/L}$ after six hours ($p<0.01$, figure 4B), which is probably due to insulin infusion. With lipid infusion, C2 concentrations decrease during the first hour (5.7 ± 0.6 to 4.2 ± 0.4 $\mu\text{mol/L}$, $p<0.01$) and subsequently tended to increase again ($p=0.052$ compared to $t=60$), reaching concentrations of 6.3 ± 0.7 $\mu\text{mol/L}$ after 6 hours which are comparable to baseline values ($p>0.05$). Infusion of carnitine in addition to lipids prevented the decrease in C2 concentrations after one hour resulting in significantly higher C2 levels compared to CON and LIPID. After six hours, plasma acetylcarnitine concentrations were increased compared to baseline ($p<0.01$, figure 4B).

Figure 3. Glucose oxidation (A), Lipid oxidation (B), respiratory exchange ratio (RER) (C) and metabolic flexibility expressed as delta RER (D) assessed during a hyperinsulinemic-euglycemic clamp in CON, LIPID and LIPID+CAR. Black bars represent CON, white bars LIPID and grey bars represent LIPID+CAR. Basal clamp is measured from $t=-30$ - $t=0$, mid-way from $t=120$ - 150 and clamp from $t=330$ - 360 . Data are expressed as mean + SEM. *, Significantly different from CON ($P<0.05$).

C3 and short-chain acylcarnitines (C3 until C5) both showed a time*treatment interaction ($p<0.05$) and were similar between groups in the basal state after an overnight fast. C3 and short-chain acylcarnitine decreased in the CON and LIPID condition after six hours ($p<0.01$), whereas increased C3 and short-chain acylcarnitines upon carnitine infusion were found after 6 hours compared to the CON and LIPID condition ($p<0.01$, figure 4C). A time*treatment interaction was found for both medium- and long-chain acylcarnitines ($p<0.01$). Plasma medium and long-chain acylcarnitines were not different between groups after an overnight fast. Insulin stimulation resulted in a reduction in medium- as well as long-chain acylcarnitines in the control group in time ($p<0.05$). In contrast, medium- and long-chain acylcarnitines increased upon lipid infusion after six hours ($p<0.05$, figure 4D + E). This increase was

even more pronounced when combining lipid infusion with carnitine infusion for both medium- as long-chain acylcarnitines ($p < 0.01$, figure 4D + E).

Figure 4. Plasma free carnitine availability (A), acetylcarnitine (B), C₃ acylcarnitine (C), medium chain acylcarnitines and long-chain acylcarnitine concentrations (E) measured at baseline ($t=0$), after one hour ($t=60$) and at the of the 6-hour hyperinsulinemic-euglycemic clamp ($t=360$). Black dots represent the control group, white dots the LIPID group and light grey lines the LIPID+CAR group. Data are expressed as means $\pm$ SEM. * significantly different from CON ($p < 0.05$).

Skeletal muscle acylcarnitines profiles

Skeletal muscle acylcarnitine profiles were determined in muscle biopsies taken after the 6-hour hyperinsulinemic-euglycemic clamp in the CON, LIPID and LIPID+CAR group, and compared to a muscle biopsy taken after an overnight fast in the basal condition (taken in the LIPID+CAR condition). Because of Bonferroni correction for multiple testing, p -values of 0.0125 were considered statistically significant for the comparisons of insulin-stimulated states with the basal state. No differences in skeletal muscle free carnitine availability ($p=0.901$) and acetylcarnitine concentrations ($p=0.786$) were found after 6-hours of infusion between groups (figure 5A and 5B), and the values obtained after 6 hours were not different from baseline ($p > 0.0125$). Short-chain acylcarnitine species reduced upon 6-hours of insulin infusion in CON trial compared to basal ($p=0.01$), but this decrease was blunted upon LIPID and LIPID+CARN compared to CON, resulting in a tendency towards higher concentrations upon LIPID and LIPID+CARN compared to CON ($p=0.10$, figure 5C). Medium and long-chain acylcarnitines seemed to be decreased in CON compared to baseline after 6 hours of insulin infusion, but this did not reach significance after Bonferroni correction for multiple testing ($p > 0.0125$, figure 5D+E). No

difference in medium and long-chain acylcarnitines were found after 6-hours of infusion between groups (figure 5D+E).

Figure 5. Skeletal muscle acylcarnitine concentrations measured in biopsies at the end of the 6-hour hyperinsulinemic-euglycemic clamp. Black bars represent the control group, white bars the LIPID group and light grey bars the LIPID+CAR group. Dar grey bars represent the pre-clamp muscle biopsy. Data are expressed as means $\pm$ SEM. * significantly different from PRE ($p < 0.0125$ after Bonferroni correction for multiple testing), tending to be different from CON ($p < 0.10$).

DISCUSSION

In the present study, we aimed to investigate whether free carnitine availability could alleviate lipid-induced insulin resistance. By increasing free carnitine availability via intravenous infusion of L-carnitine we hypothesized that free carnitine availability in skeletal muscle tissue increases and thereby could prevent the development of lipid-induced insulin resistance and metabolic inflexibility during acute lipid infusion.

In the current study, the intravenous administration of carnitine increased plasma free carnitine concentrations to 183 $\mu\text{mol/L}$. These values exceed normal reference values (22.30-54.80 $\mu\text{mol/L}$) indicating a state of hypercarnitinemia and thus increased free carnitine availability. Earlier studies have also reported hypercarnitinemia upon intravenous carnitine infusion with similar carnitine infusion dosages (18, 29).

Although plasma hypercarnitinemia occurred, surprisingly no differences in skeletal muscle free carnitine availability were found upon carnitine infusion. The uptake of carnitine into the skeletal muscle cells is regulated via the sodium dependent organic cation transporter 2 (OCTN2) and tightly regulated by the sarcolemmal Na^+/K^+ ATPase pump activity (30-34). Indeed, inhibition of this Na^+/K^+ ATPase pump activity has been shown to decrease carnitine uptake in isolated skeletal muscle cells illustrating the importance of the Na^+/K^+ ATPase pump activity in this sodium dependent uptake of carnitine (32, 35). Insulin is known to facilitate this carnitine uptake into the skeletal muscle (31, 33, 36) by increasing the activity of the sarcolemmal Na^+/K^+ ATPase pump. Therefore, carnitine infusion together with hyperinsulinemia (40 $\text{mU/m}^2/\text{min}$) was expected to increase skeletal muscle free carnitine levels. A possible explanation for the lack of increase in skeletal muscle free carnitine and acetylcarnitine in the current study could be that due to the development of lipid-induced insulin resistance, the expected insulin facilitated increase in Na^+/K^+ ATPase pump activity may have been absent, thereby not leading to enhanced sodium mediated co-transport of carnitine into the skeletal muscle (31, 32, 37-39). Unfortunately, we did not perform a clamp with intravenous infusion of carnitine alone, without additional lipid infusion. The latter could have disclosed whether lipid infusion indeed hampered carnitine uptake versus insulin infusion alone. Therefore, future studies are needed to investigate whether a lipid overload (such as with intravenous lipid infusion) could indeed decrease the insulin mediated uptake of free carnitine in skeletal muscle tissue.

It is also possible that the insulin infusion rate ($40 \text{ mU/m}^2/\text{min}$) used in the present study was insufficient to cause free carnitine uptake in skeletal muscle tissue. It is known that an insulin infusion rate of $5 \text{ mU/m}^2/\text{min}$ did not affect carnitine uptake in skeletal muscle but high concentrations of insulin infusion ($105 \text{ mU/m}^2/\text{min}$) did result in a 15% increases in skeletal muscle total carnitine content (9, 33), and elevated OCTN2 mRNA expression was associated with the increase in total skeletal muscle carnitine content (33). Using lower insulin infusion rates (30 and $55 \text{ mU/m}^2/\text{min}$), Stephens et al. reported that at least an infusion rate of $55 \text{ mU/m}^2/\text{min}$ was needed to stimulate skeletal muscle carnitine uptake. It could therefore be suggested that the insulin infusion rate of $40 \text{ mU/m}^2/\text{min}$, which we used here, was not high enough to increase total carnitine content in muscle tissue (40). Future studies will have to address this and more precisely determine the degree of insulin stimulation that is necessary to result in an elevated muscle carnitine content.

The increase in lipid availability as result of lipid infusion lead to strongly elevated plasma free fatty acid levels, as reported before (3, 28). It was previously reported that due to this rise in FFA levels, glucose infusion rates (GIR), insulin sensitivity and metabolic flexibility decreases after 2-4 hours of lipid infusion (3, 4, 28, 41-43). Indeed, we found that glucose infusion rate and M-value both decreased by approximately 50% indicating a marked induction of insulin resistance upon lipid infusion. Furthermore, carbohydrate oxidation was reduced and lipid oxidation increased in the insulin stimulated state, reflecting a blunted metabolic flexibility upon insulin stimulation. However, these changes were similar in the conditions with or without infusion of carnitine. This lack of effect of additional carnitine infusion on lipid-induced insulin resistance is in line with our hypothesis that changes in skeletal muscle free carnitine, which were unfortunately not achieved here, are necessary to rescue lipid-induced insulin resistance. In contrast to our findings, beneficial effects of L-Carnitine infusion has been reported previously in patients with type 2 diabetes. Thus, in these studies, intravenous infusion of L-carnitine during a hyperinsulinemic-euglycemic clamp (40 mU/kg/min) was shown to improve whole-body glucose disposal (17, 18). Furthermore, Mingrone et al. (17) reported enhanced insulin stimulated glucose oxidation upon carnitine infusion during a clamp (40 mU/kg/min), reflecting improved metabolic flexibility. However, in these studies, skeletal muscle free carnitine availability is not reported. It should be noted that in these studies, no lipid infusion was used and increases in skeletal muscle free carnitine is therefore more likely. Therefore, the additional lipid infusion and the

concomitant absence of increased skeletal muscle free carnitine availability in the current study might be responsible for the contrasting result between our study and previous research. Whether improved skeletal muscle free carnitine availability indeed underlies the beneficial metabolic effects that were reported previously, remains elusive since skeletal muscle free carnitine availability was not measured in these studies. Since we aimed to test the hypothesis that carnitine could prevent lipid-induced insulin resistance, we used lipid infusion as a model to simulate insulin resistance by lipid overload in lean healthy subjects; obviously, this model cannot be directly compared to the insulin resistance that is observed in type 2 diabetes.

Stephens et al. reported previously that in the resting state 30% of the total carnitine pool is present as acetylcarnitine, increasing to 70% after exercise at 75% of VO_{2max} (9). This increase is in line with the increase in *in vivo* acetylcarnitine concentration we measured after exercise using MRS. However, no correlation was found between acetylcarnitine formation capacity and insulin sensitivity indicating that the capacity to form acetylcarnitine could not predict lipid-induced insulin resistance. However, since the intra-individual variation was very small, this might explain the lack of correlation.

In the current study, plasma acetylcarnitine concentrations were reduced upon insulin stimulation in the control trial. Next to acetylcarnitine levels, reduced short-, medium- and long-chain acylcarnitine levels have been reported in situations of hyperinsulinemia. We here confirmed this reduction in short-, medium- and long-chain acylcarnitines levels upon insulin infusion. This decreases in acylcarnitine profiles are likely to reflect a decreased lipid oxidation caused by the hyperinsulinemia, as previously reported (44, 45). Indeed, decreased lipid oxidation and increased glucose oxidation were observed upon hyperinsulinemia in the control trial. Lipid infusion increased plasma acetylcarnitine, medium- and long-chain acylcarnitines, probably reflecting increased efflux of β -oxidation intermediates by tissues such as liver and muscle (46, 47). Lipid infusion increases carbon load through the β -oxidation. As a consequence, oxidation is fueled by lipids and could therefore explain the elevated acetylcarnitine concentrations in the plasma. The main contributor to the plasma acetylcarnitine elevations might be increased production by the β -oxidation and subsequently release of acetylcarnitine by the liver as indicated by earlier studies using a porcine animal model or human volunteers to assess trans organ acylcarnitine fluxes (47, 48). In line with this, plasma C4OH-carnitine concentration increased upon lipid infusion. This specific acylcarnitine

species is mainly derived from the ketone body beta-hydroxybutyrate, which is primarily produced in the liver. Plasma C3 acylcarnitines and the sum of plasma short-chain acylcarnitines (C3 to C5) did not change upon lipid infusion contrary to the other acylcarnitine species. Since C3 is mainly derived from branches-chain amino acids this might explain the different kinetics. With additional intravenous carnitine infusion (LIPID+CAR), an even more pronounced increase in plasma acetylcarnitine, medium- and long-chain acylcarnitines compared to only lipid infusion, was observed. Similarly, C3 and short-chain acylcarnitine were also increased upon combined lipid + carnitine infusion compared to lipid infusion alone ($p < 0.05$). As plasma acylcarnitine concentrations are significantly higher upon carnitine infusion, these data indicate the necessity of free carnitine availability in the formation of acylcarnitine species.

Surprisingly, skeletal muscle acetylcarnitine concentrations remained unaffected by lipid infusion as well as lipid combined with carnitine infusion. Contrary, Tsintzas et al. reported increased skeletal muscle acetylcarnitine concentrations upon lipid infusion. Although we cannot provide a direct explanation for this discrepancy, the more than two-fold higher plasma FFA concentration in the study of Tsintzas might be of relevance. Future research is necessary to unravel this difference.

Furthermore, skeletal muscle short-acylcarnitine levels decreased upon insulin infusion in the control trial. Medium- and long-chain acylcarnitine seemed to decrease as well, although not reaching significance. Insulin reduces lipolysis resulting in decreased plasma FFA availability, and as a consequence, glucose oxidation increases. The decreases in skeletal muscle acylcarnitine profiles upon insulin therefore probably reflects this decreased FFA availability resulting in a transition of lipid towards glucose oxidation induced by hyperinsulinemia (44, 45). Lipid infusion increased plasma FFA concentrations despite high insulin concentration. Subsequently, the decrease in short- and medium-chain acylcarnitines in skeletal muscle tissue as found in the control trial was blunted upon lipid infusion, which may indicate higher skeletal muscle lipid oxidation rates. Remarkably, the reduction in long-chain acylcarnitine species was not blunted by lipid infusion; although we cannot provide a direct explanation for this effect, it could be speculated that during acute lipid overload accumulation of beta-oxidation intermediates does mainly happen at later passages through the β -oxidation.

In conclusion, lipid infusion strongly increased plasma FFA levels and resulted in a

hampered metabolic flexibility and insulin sensitivity. Intravenous infusion of carnitine in addition to lipid elevated plasma free carnitine availability. However, against expectations, carnitine infusion did not increase skeletal muscle free carnitine availability possibly due to insulin resistance of the OCTN2 receptor involved in skeletal muscle carnitine uptake. In line with the lack of increase in skeletal muscle free carnitine availability, lipid-induced metabolic inflexibility and insulin resistance could not be rescued by carnitine administration.

REFERENCES

1. Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes*. 2000;49(5):677-83.
2. Perseghin G, Ghosh S, Gerow K, Shulman GI. Metabolic defects in lean nondiabetic offspring of NIDDM parents: a cross-sectional study. *Diabetes*. 1997;46(6):1001-9.
3. Meex RC, Phielix E, Moonen-Kornips E, Schrauwen P, Hesselink MK. Stimulation of human whole-body energy expenditure by salsa is fueled by higher lipid oxidation under fasting conditions and by higher oxidative glucose disposal under insulin-stimulated conditions. *The Journal of clinical endocrinology and metabolism*. 2011;96(5):1415-23.
4. Phielix E, Meex R, Ouwens DM, Sparks L, Hoeks J, Schaart G, et al. High oxidative capacity due to chronic exercise training attenuates lipid-induced insulin resistance. *Diabetes*. 2012;61(10):2472-8.
5. Bieber LL. Carnitine. *Annual review of biochemistry*. 1988;57:261-83.
6. McGarry JD, Sen A, Esser V, Woeltje KF, Weis B, Foster DW. New insights into the mitochondrial carnitine palmitoyltransferase enzyme system. *Biochimie*. 1991;73(1):77-84.
7. Muoio DM, Noland RC, Kovalik JP, Seiler SE, Davies MN, DeBalsi KL, et al. Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. *Cell metabolism*. 2012;15(5):764-77.
8. Noland RC, Koves TR, Seiler SE, Lum H, Lust RM, Ilkayeva O, et al. Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control. *The Journal of biological chemistry*. 2009;284(34):22840-52.
9. Stephens FB, Constantin-Teodosiu D, Greenhaff PL. New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *The Journal of physiology*. 2007;581(Pt 2):431-44.
10. Power RA, Hulver MW, Zhang JY, Dubois J, Marchand RM, Ilkayeva O, et al. Carnitine revisited: potential use as adjunctive treatment in diabetes. *Diabetologia*. 2007;50(4):824-32.
11. Lindeboom L, Nabuurs CI, Hoeks J, Brouwers B, Phielix E, Kooi ME, et al. Long-echo time MR spectroscopy for skeletal muscle acetylcarnitine detection. *The Journal of clinical investigation*. 2014;124(11):4915-25.

12. Ringseis R, Keller J, Eder K. Role of carnitine in the regulation of glucose homeostasis and insulin sensitivity: evidence from in vivo and in vitro studies with carnitine supplementation and carnitine deficiency. *European journal of nutrition*. 2012;51(1):1-18.
13. Malaguarnera M, Gargante MP, Russo C, Antic T, Vacante M, Malaguarnera M, et al. L-carnitine supplementation to diet: a new tool in treatment of nonalcoholic steatohepatitis—a randomized and controlled clinical trial. *The American journal of gastroenterology*. 2010;105(6):1338-45.
14. Rahbar AR, Shakerhosseini R, Saadat N, Taleban F, Pordal A, Goldestan B. Effect of L-carnitine on plasma glycemic and lipidemic profile in patients with type II diabetes mellitus. *European journal of clinical nutrition*. 2005;59(4):592-6.
15. Molfino A, Cascino A, Conte C, Ramaccini C, Rossi Fanelli F, Laviano A. Caloric restriction and L-carnitine administration improves insulin sensitivity in patients with impaired glucose metabolism. *JPEN Journal of parenteral and enteral nutrition*. 2010;34(3):295-9.
16. Capaldo B, Napoli R, Di Bonito P, Albano G, Sacca L. Carnitine improves peripheral glucose disposal in non-insulin-dependent diabetic patients. *Diabetes research and clinical practice*. 1991;14(3):191-5.
17. Mingrone G, Greco AV, Capristo E, Benedetti G, Giancaterini A, De Gaetano A, et al. L-carnitine improves glucose disposal in type 2 diabetic patients. *Journal of the American College of Nutrition*. 1999;18(1):77-82.
18. Giancaterini A, De Gaetano A, Mingrone G, Gniuli D, Liverani E, Capristo E, et al. Acetyl-L-carnitine infusion increases glucose disposal in type 2 diabetic patients. *Metabolism: clinical and experimental*. 2000;49(6):704-8.
19. Dempster P, Aitkens S. A new air displacement method for the determination of human body composition. *Medicine and science in sports and exercise*. 1995;27(12):1692-7.
20. Vink RG, Roumans NJ, Arkenbosch LA, Mariman EC, van Baak MA. The effect of rate of weight loss on long-term weight regain in adults with overweight and obesity. *Obesity (Silver Spring, Md)*. 2016;24(2):321-7.
21. Siri WE. Body composition from fluid spaces and density: analysis of methods. 1961. *Nutrition (Burbank, Los Angeles County, Calif)*. 1993;9(5):480-91; discussion , 92.
22. Bilet L, van de Weijer T, Hesselink MK, Glatz JF, Lamb HJ, Wildberger J, et al. Exercise-induced modulation of cardiac lipid content in healthy lean young men. *Basic research in cardiology*. 2011;106(2):307-15.

23. Vanhamme L, van den Boogaart A, Van Huffel S. Improved method for accurate and efficient quantification of MRS data with use of prior knowledge. *Journal of magnetic resonance* (San Diego, Calif : 1997). 1997;129(1):35-43.
24. Peronnet F, Massicotte D. Table of nonprotein respiratory quotient: an update. *Can J Sport Sci.* 1991;16(1):23-9.
25. Bergstrom J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance. *Acta physiologica Scandinavica.* 1967;71(2):140-50.
26. van Vlies N, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, et al. Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. *The Biochemical journal.* 2005;387(Pt 1):185-93.
27. Vreken P, van Lint AE, Bootsma AH, Overmars H, Wanders RJ, van Gennip AH. Quantitative plasma acylcarnitine analysis using electrospray tandem mass spectrometry for the diagnosis of organic acidemias and fatty acid oxidation defects. *Journal of inherited metabolic disease.* 1999;22(3):302-6.
28. Hoeks J, Mensink M, Hesselink MK, Ekroos K, Schrauwen P. Long- and medium-chain fatty acids induce insulin resistance to a similar extent in humans despite marked differences in muscle fat accumulation. *The Journal of clinical endocrinology and metabolism.* 2012;97(1):208-16.
29. Natali A, Santoro D, Brandi LS, Faraggiana D, Ciociaro D, Pecori N, et al. Effects of acute hypercarnitinemia during increased fatty substrate oxidation in man. *Metabolism: clinical and experimental.* 1993;42(5):594-600.
30. Clausen T. Na⁺-K⁺ pump regulation and skeletal muscle contractility. *Physiological reviews.* 2003;83(4):1269-324.
31. Deachapunya C, Palmer-Densmore M, O'Grady SM. Insulin stimulates transepithelial sodium transport by activation of a protein phosphatase that increases Na-K ATPase activity in endometrial epithelial cells. *The Journal of general physiology.* 1999;114(4):561-74.
32. Rebouche CJ. Carnitine movement across muscle cell membranes. *Studies in isolated rat muscle. Biochimica et biophysica acta.* 1977;471(1):145-55.
33. Stephens FB, Constantin-Teodosiu D, Laithwaite D, Simpson EJ, Greenhaff PL. Insulin stimulates L-carnitine accumulation in human skeletal muscle. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2006;20(2):377-9.

34. Tamai I, Ohashi R, Nezu J, Yabuuchi H, Oku A, Shimane M, et al. Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. *The Journal of biological chemistry*. 1998;273(32):20378-82.
35. Georges B, Le Borgne F, Galland S, Isoir M, Ecosse D, Grand-Jean F, et al. Carnitine transport into muscular cells. Inhibition of transport and cell growth by mildronate. *Biochemical pharmacology*. 2000;59(11):1357-63.
36. Pochini L, Scalise M, Galluccio M, Indiveri C. OCTN cation transporters in health and disease: role as drug targets and assay development. *Journal of biomolecular screening*. 2013;18(8):851-67.
37. Record RD, Froelich LL, Vlahos CJ, Blazer-Yost BL. Phosphatidylinositol 3-kinase activation is required for insulin-stimulated sodium transport in A6 cells. *The American journal of physiology*. 1998;274(4 Pt 1):E611-7.
38. Rodriguez-Commes J, Isales C, Kalghati L, Gasalla-Herraiz J, Hayslett JP. Mechanism of insulin-stimulated electrogenic sodium transport. *Kidney international*. 1994;46(3):666-74.
39. Ragolia L, Cherpalis B, Srinivasan M, Begum N. Role of serine/threonine protein phosphatases in insulin regulation of Na⁺/K⁺-ATPase activity in cultured rat skeletal muscle cells. *The Journal of biological chemistry*. 1997;272(38):23653-8.
40. Stephens FB, Constantin-Teodosiu D, Laithwaite D, Simpson EJ, Greenhaff PL. A threshold exists for the stimulatory effect of insulin on plasma L-carnitine clearance in humans. *American journal of physiology Endocrinology and metabolism*. 2007;292(2):E637-41.
41. Boden G, Jadali F, White J, Liang Y, Mozzoli M, Chen X, et al. Effects of fat on insulin-stimulated carbohydrate metabolism in normal men. *The Journal of clinical investigation*. 1991;88(3):960-6.
42. Hoeks J, Hesselink MK, Russell AP, Mensink M, Saris WH, Mensink RP, et al. Peroxisome proliferator-activated receptor-gamma coactivator-1 and insulin resistance: acute effect of fatty acids. *Diabetologia*. 2006;49(10):2419-26.
43. Tsintzas K, Chokkalingam K, Jewell K, Norton L, Macdonald IA, Constantin-Teodosiu D. Elevated free fatty acids attenuate the insulin-induced suppression of PDK4 gene expression in human skeletal muscle: potential role of intramuscular long-chain acyl-coenzyme A. *The Journal of clinical endocrinology and metabolism*. 2007;92(10):3967-72.
44. Consitt LA, Koves TR, Muoio DM, Nakazawa M, Newton CA, Houmard JA. Plasma acylcarnitines during insulin stimulation in humans are reflective of age-related metabolic dysfunction. *Biochemical and biophysical research communications*. 2016;479(4):868-74.

45. Soeters MR, Sauerwein HP, Duran M, Wanders RJ, Ackermans MT, Fliers E, et al. Muscle acylcarnitines during short-term fasting in lean healthy men. *Clinical science (London, England : 1979)*. 2009;116(7):585-92.
46. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell metabolism*. 2008;7(1):45-56.
47. Xu G, Hansen JS, Zhao XJ, Chen S, Hoene M, Wang XL, et al. Liver and Muscle Contribute Differently to the Plasma Acylcarnitine Pool During Fasting and Exercise in Humans. *The Journal of clinical endocrinology and metabolism*. 2016;101(12):5044-52.
48. Schooneman MG, Ten Have GA, van Vlies N, Houten SM, Deutz NE, Soeters MR. Transorgan fluxes in a porcine model reveal a central role for liver in acylcarnitine metabolism. *American journal of physiology Endocrinology and metabolism*. 2015;309(3):E256-64.
49. Pettit FH, Pelley JW, Reed LJ. Regulation of pyruvate dehydrogenase kinase and phosphatase by acetyl-CoA/CoA and NADH/NAD ratios. *Biochemical and biophysical research communications*. 1975;65(2):575-82.

SUPPLEMENTARY DATA

Supplementary Table. 1. Plasma acylcarnitines before, during and at the end of the 6-hour hyperinsulinemic-euglycemic clamp

		CON	LIPID	LIPID+CAR
C0	t=0	35.79 ± 2.02	36.32 ± 2.82	34.40 ± 1.87
	t=60	36.84 ± 2.09	36.96 ± 2.64	155.38 ± 4.94
	t=360	33.78 ± 2.04	31.55 ± 2.16	183.33 ± 6.28
C2	t=0	5.38 ± 0.71	5.65 ± 0.65	5.13 ± 0.65
	t=60	3.56 ± 0.34	4.16 ± 0.37	5.70 ± 0.62
	t=360	2.27 ± 0.10	6.31 ± 0.69	15.11 ± 1.04
C3	t=0	0.36 ± 0.03	0.35 ± 0.03	0.36 ± 0.05
	t=60	0.36 ± 0.03	0.33 ± 0.03	0.48 ± 0.06
	t=360	0.22 ± 0.02	0.17 ± 0.01	0.47 ± 0.05
C4	t=0	0.20 ± 0.01	0.18 ± 0.01	0.20 ± 0.02
	t=60	0.19 ± 0.02	0.17 ± 0.01	0.22 ± 0.02
	t=360	0.15 ± 0.01	0.14 ± 0.01	0.28 ± 0.03
C5:1	t=0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=60	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=360	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
C5	t=0	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
	t=60	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.01
	t=360	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01
C4-3OH	t=0	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.01
	t=60	0.02 ± 0.00	0.03 ± 0.00	0.05 ± 0.02
	t=360	0.01 ± 0.00	0.06 ± 0.01	0.14 ± 0.03
C6	t=0	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
	t=60	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.01
	t=360	0.02 ± 0.00	0.04 ± 0.00	0.08 ± 0.01

C5OH	t=0	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
	t=60	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	t=360	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C8	t=0	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
	t=60	0.06 ± 0.00	0.07 ± 0.01	0.08 ± 0.01
	t=360	0.03 ± 0.00	0.07 ± 0.01	0.10 ± 0.01
C3 DC	t=0	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.01
	t=60	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
	t=360	0.01 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
C10:1	t=0	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
	t=60	0.04 ± 0.01	0.09 ± 0.01	0.10 ± 0.02
	t=360	0.01 ± 0.00	0.19 ± 0.01	0.27 ± 0.02
C10	t=0	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
	t=60	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
	t=360	0.01 ± 0.00	0.04 ± 0.00	0.06 ± 0.01
C4 DC	t=0	0.04 ± 0.00	0.04 ± 0.01	0.03 ± 0.01
	t=60	0.04 ± 0.00	0.04 ± 0.01	0.03 ± 0.01
	t=360	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
C5 DC	t=0	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
	t=60	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
	t=360	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
C12:1	t=0	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01
	t=60	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	t=360	0.00 ± 0.00	0.03 ± 0.00	0.05 ± 0.00
C12	t=0	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01
	t=60	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
	t=360	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.00

C6 DC	t=0	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
	t=60	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=360	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C12:1OH	t=0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=60	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=360	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
C12OH	t=0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=60	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=360	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C53M3OH DC	t=0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=60	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=360	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C14:2	t=0	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	t=60	0.01 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
	t=360	0.00 ± 0.00	0.12 ± 0.02	0.21 ± 0.04
C14:1	t=0	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
	t=60	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01
	t=360	0.01 ± 0.00	0.06 ± 0.01	0.10 ± 0.02
C14	t=0	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	t=60	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=360	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
C8 DC	t=0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=60	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=360	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C14:1OH	t=0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=60	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=360	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00

C14OH	t=0	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
	t=60	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=360	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C16:1	t=0	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	t=60	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=360	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C16	t=0	0.08 ± 0.01	0.08 ± 0.00	0.08 ± 0.01
	t=60	0.07 ± 0.01	0.08 ± 0.00	0.07 ± 0.01
	t=360	0.03 ± 0.00	0.07 ± 0.00	0.08 ± 0.01
C10 DC	t=0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=60	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	t=360	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
C16:1OH	t=0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=60	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=360	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C16OH	t=0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=60	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=360	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18:2	t=0	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
	t=60	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
	t=360	0.02 ± 0.00	0.13 ± 0.01	0.15 ± 0.02
C18:1	t=0	0.08 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
	t=60	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
	t=360	0.04 ± 0.00	0.08 ± 0.01	0.09 ± 0.00
C18	t=0	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
	t=60	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
	t=360	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00

C18:2OH	t=0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=60	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=360	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C18:1OH	t=0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=60	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=360	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
C18OH	t=0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=60	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	t=360	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Supplementary Table. 2. Skeletal muscle acylcarnitine concentrations (in biopsies) before (only LIPID+CAR trial) and after the 6-hour hyperinsulinemic-euglycemic clamp

	CON after	LIPID after	LIPID+CAR before	LIPID+CAR after
C0	4865.56 ± 1081.90	5391.99 ± 685.81	4559.25 ± 761.19	5402.57 ± 998.28
C2	476.28 ± 89.07	556.02 ± 83.27	822.57 ± 257.62	564.47 ± 111.55
C3	6.71 ± 1.43	6.84 ± 0.82	8.06 ± 1.30	7.15 ± 0.98
C4	2.86 ± 0.74	5.26 ± 1.02	8.55 ± 1.94	4.96 ± 1.44
C5:1	0.83 ± 0.33	0.63 ± 0.15	0.64 ± 0.23	0.43 ± 0.13
C5	2.59 ± 0.76	1.89 ± 0.40	3.78 ± 1.08	1.74 ± 0.45
C4-3OH	1.14 ± 0.20	9.56 ± 2.18	6.26 ± 2.80	10.39 ± 2.90
C6	0.79 ± 0.21	3.76 ± 1.29	8.15 ± 3.08	3.58 ± 1.58
C8:1	0.44 ± 0.13	1.79 ± 0.33	0.77 ± 0.18	1.18 ± 0.27
C8	0.66 ± 0.13	2.30 ± 0.81	4.47 ± 1.60	2.22 ± 0.84
C4DC	1.12 ± 0.13	1.45 ± 0.17	1.59 ± 0.29	1.48 ± 0.25
C10	0.51 ± 0.11	0.95 ± 0.24	2.70 ± 0.87	1.03 ± 0.32
C12:1	0.30 ± 0.07	0.44 ± 0.11	1.20 ± 0.45	0.52 ± 0.14
C12	0.87 ± 0.19	1.07 ± 0.22	3.84 ± 1.46	1.14 ± 0.30
C14:2	0.63 ± 0.13	2.35 ± 0.61	2.06 ± 0.63	2.38 ± 0.81
C14:1	1.67 ± 0.40	2.30 ± 0.48	8.44 ± 3.30	2.53 ± 0.75
C14	2.54 ± 0.58	2.32 ± 0.38	9.35 ± 3.60	2.77 ± 0.52
C16:2	0.85 ± 0.20	1.92 ± 0.36	1.75 ± 0.54	2.01 ± 0.43
C16:1	5.18 ± 1.30	3.55 ± 0.30	10.65 ± 3.43	4.10 ± 0.68
C16	14.40 ± 3.05	9.78 ± 1.27	28.43 ± 9.44	11.28 ± 1.53
C18:2	12.62 ± 2.58	15.57 ± 1.49	16.10 ± 3.55	19.56 ± 3.24
C18:1	31.96 ± 7.33	19.77 ± 1.81	47.24 ± 10.62	27.89 ± 4.38
C18	4.75 ± 0.87	3.88 ± 0.54	10.27 ± 2.88	4.73 ± 0.72
C20:2	0.11 ± 0.02	0.09 ± 0.01	0.20 ± 0.08	0.12 ± 0.01
C20:1	0.20 ± 0.03	0.15 ± 0.02	0.43 ± 0.14	0.19 ± 0.03
C20	0.06 ± 0.01	0.14 ± 0.02	0.18 ± 0.06	0.15 ± 0.03

Chapter 4

Carnitine supplementation improves metabolic flexibility and skeletal muscle acetylcarnitine formation

Yvonne M.H. Bruls

Marlies de Ligt

Lucas Lindeboom

Esther Phielix

Bas Havekes

Joachim E. Wildberger

Matthijs K. Hesselink

Patrick Schrauwen

Vera B. Schrauwen-Hinderling

ABSTRACT

Objective: Insulin resistant subjects and patients with type 2 diabetic are characterized by disturbed glucose homeostasis and decreased metabolic flexibility. Recent evidence indicates that low carnitine availability may be underlying the reduced metabolic flexibility and impaired glucose tolerance. Here, we investigated whether carnitine supplementation improves metabolic flexibility and insulin sensitivity in volunteers with impaired glucose tolerance (IGT).

Methods: Eleven IGT volunteers followed a 36-day placebo- and L-carnitine treatment (2 g/day) in a randomized, 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 (¹H-MRS, TE=500 ms) in the resting state (7:00 AM and 5:00 PM). To stimulate acetylcarnitine formation as parameter for free carnitine availability, a 30-minute cycling exercise was performed at 70% of the volunteers predetermined maximal output (W_{max}) followed by acetylcarnitine determination by ¹H-MRS. Twelve normal glucose tolerant (NGT) volunteers were included without any intervention as control group.

Results: Metabolic flexibility was lower in IGT compared to NGT (0.07 ± 0.01 and 0.10 ± 0.01 AU, $p=0.22$), but was completely restored upon carnitine supplementation (0.10 ± 0.1 , $p<0.05$) as measured during a hyperinsulinemic-euglycemic clamp. Similar results on metabolic flexibility were found in response to a high-energy meal test ($p<0.05$). Carnitine supplementation also enhanced the increase in resting skeletal muscle acetylcarnitine concentrations over the day (delta of 7:00 AM versus 5:00 PM: 1.57 ± 0.33 and 0.67 ± 0.18 mmol/kgww upon carnitine and placebo respectively, $p<0.05$). Besides, post-exercise acetylcarnitine concentrations increased compared to placebo (1.62 ± 0.27 and 1.08 ± 0.20 mmol/kgww respectively, ($p<0.05$). Whole-body insulin sensitivity was not affected by carnitine supplementation ($p>0.05$).

Conclusion: Carnitine supplementation completely restored metabolic flexibility in impaired glucose tolerant subjects. A larger increase over the day in resting skeletal muscle acetylcarnitine concentration as well as the improved capacity to form acetylcarnitine with exercise may be underlying the beneficial effects on metabolic flexibility.

INTRODUCTION

Metabolic flexibility is defined as the capacity to switch from predominantly fat oxidation while fasting, to carbohydrate oxidation in the insulin-stimulated state and vice versa (1). 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 and/or prevent disturbed glucose homeostasis.

It has recently been suggested that the formation of acetylcarnitine is crucial in maintaining metabolic flexibility, resulting in improved glucose homeostasis (3-6). Acetylcarnitine is synthesized by the conjugation of acetyl-CoA and free carnitine, mediated via the enzyme carnitine acyltransferase (CrAT) (3-6). This formation of acetylcarnitine acts as a buffering system to regulate the acetyl-CoA pool, and thus to prevent intra-mitochondrial accumulation of acetyl-CoA. The buffering of acetyl-CoA is especially important under conditions of excessive substrate supply to the TCA cycle, such as during (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 mitochondrial indecision. This indecision refers to the fact that the regulation as to which substrate prevails for oxidation is overruled by signals of substrate overabundance. Thus the inflow of acetyl-CoA from fatty acid oxidation can result in unresponsiveness to the regulatory signal of insulin to switch from fatty acid oxidation to glucose oxidation. Therefore, metabolic inflexibility at the mitochondrial level occurs (6).

In accordance, knocking-out the CrAT enzyme in mice results in lower acetylcarnitine formation and blunted metabolic flexibility. Furthermore, CrAT gain-of function studies in human primary myotubes showed elevated mitochondrial acetylcarnitine efflux, indicating enhanced acetylcarnitine formation with higher CrAT enzyme activity (3), resulting in higher PDH activity and metabolic flexibility (3). These results suggest an important role for acetylcarnitine formation in maintaining metabolic flexibility.

When there is excessive substrate supply to the mitochondria, a mismatch between β -oxidation and TCA cycle occurs. Typically this mismatch results 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 due to the accumulation of long chain acylcarnitines may diminish the capacity to form acetylcarnitine further, as free carnitine availability is crucial in maintaining the proposed mitochondrial rescue mechanism of acetylcarnitine formation (3, 4, 9). Therefore, next to low CrAT activity, also a decreased carnitine availability may be underlying the development of metabolic inflexibility. For example, it was found that diabetic mice (BAP-agouti transgenic mice) were characterized by reduced acetylcarnitine 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 in these diabetic mice (9). Furthermore, Noland et al. (4) found that feeding rats a 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 capacity to form acetylcarnitine is underlying the beneficial effects of carnitine supplementation on metabolic flexibility and concomitantly improved glucose tolerance in humans. 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 HOMA-IR index (12, 13) or glucose area under the curve after an oral glucose tolerance test (15). These results seem to suggest that also in humans, carnitine supplementation may enhance the capacity to form acetylcarnitine and improve metabolic flexibility. 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 acetylcarnitine concentrations in skeletal muscle *in vivo* (16). This technique provides the unique opportunity to non-invasively and dynamically investigate the role of acetylcarnitine 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 acetylcarnitine formation and improves metabolic flexibility and insulin sensitivity in individuals with impaired glucose tolerance.

METHODS

Subjects

Twelve normal glucose tolerant (NGT) and eleven impaired glucose tolerant (IGT) individuals participated in this study. Impaired glucose tolerance was defined as a plasma glucose level between 7.8-11.1 mmol/L 2-hours after a 75 gram glucose bolus provided during an oral glucose tolerance test (OGTT). NGT was defined as plasma glucose levels <7.8 mmol/L and >11.1 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 hours a week, impaired renal and/or kidney function, uncontrolled hypertension, history of cardiovascular disease, MRI contra-indications, use of medication known to interfere with glucose homeostasis and being vegetarian, which can affect the whole body carnitine status. Before written informed consent was obtained, the protocol was explained in full, including potential risks. 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 NTC02072759.

Experimental design

The study was set up in a double blind, randomized cross-over design in volunteers with IGT. Normal glucose tolerant (NGT) volunteers served as control group for baseline comparison. IGT participants were randomly assigned to one of the two periods of the intervention trial: placebo or carnitine (2000 mg/day of L-carnitine). After a wash-out period of at least 4 weeks volunteers followed the other treatment. At the beginning of each period, a fasted blood sample was obtained after which supplementation was started and continued for 36 days (figure 1). Participants reported to the laboratory on a weekly basis to obtain a fasting blood sample and to supply supplements for the next week. Participants were asked to maintain their normal diet and physical activity pattern during participation in the study.

Figure 1. Outline of the double blind, randomized cross-over design.

On day 30, participants reported to the laboratory at 6:00 AM after an overnight fast. A meal test was performed to investigate metabolic flexibility by indirect calorimetry upon a high-energy breakfast provided to the volunteers at 8:00 AM at the laboratory. *In vivo* skeletal muscle acetylcarnitine concentrations were measured via proton magnetic resonance spectroscopy (^1H -MRS) (16) before and several time points after this breakfast. On a separate day (day 33), a biopsy was taken and a hyperinsulinemic-euglycemic clamp was performed to measure peripheral insulin sensitivity. Finally, on a third test day (day 36), participants came to the laboratory at 4:30 PM. After consumption of a light lunch at 12:00 AM, participants remained fasted for the following 5 hours. At 5:00 PM, participants were positioned in the MR scanner and skeletal muscle acetylcarnitine concentrations were measured using long-TE ^1H -MRS at rest and directly after a 30-minute cycling exercise at 70% of the subjects predetermined maximal output (W_{max}). NGT participants were subjected to the same measurements but without any intervention trial (no placebo and carnitine).

Treatment / Carnitine supplementation

L-Carnitine tartrate (NOW foods, Bloomingdale, IL, USA) was taken orally for 36 days (2000 mg carnitine/d); 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. Compliance was checked by measuring acylcarnitines levels in the plasma before the start of the intervention at day 1 and on the morning of the clamp (day 33). Placebo capsules (Pharmacy Radboud UMC, Nijmegen, The Netherlands) containing 500-mg micro cellulose were provided in an identical regimen.

VO_{2max} and body composition

On a separate day, before the start of the intervention trials, participants underwent an incremental cycling test on a stationary bike to determine maximal oxygen uptake (VO_{2max}) and maximal power output (W_{max}) for characterization of the participants. On the same day, body composition was determined by DXA (DXA, discovery A; Hologic).

Meal test

On day 30, participants reported to the laboratory at 6:00 AM after an overnight fast. To standardize the diet, all participants were provided with a standardized chicken-rice dinner for consumption at home on day 29. Furthermore, participants were asked to refrain from strenuous physical activity three days prior to the meal test. After arrival at the laboratory, subjects were bed-rested in supine position and a catheter was inserted in the median cubital vein for repetitive blood sampling throughout the test day. A first 10-ml blood sample was taken in the fasted condition before the meal. While resting, indirect calorimetry (Ventilated hood, Omnicol, Maastricht Instruments, Maastricht University) was performed to determine substrate oxidation at baseline (t=-90). Subsequently, a baseline proton magnetic resonance spectroscopy (¹H-MRS) scan was performed, using long echo times (TE) to quantify *in vivo* skeletal muscle acetylcarnitine (=30 min) as previously reported (16). After these measurements, subjects consumed a high-energy breakfast. The high-energy breakfast consisted of sausage rolls (65.5% energy from fat, 31.6% energy of carbohydrates, and 6.9% energy from proteins). The total energy content of the breakfast was equal to 50% of the participants required daily energy intake. Daily energy intake was calculated on the basis of lean body mass as measured during the screening visit according to the Cunningham equation (18). A correction factor of 1.3 was applied for physical activity. ¹H-MRS measurements were repeated directly after the breakfast (t=30 min), 2.5 hours (t=150 min) and 5.5 hours (t=330 min) after breakfast. Blood samples were drawn before each MR spectroscopy measurement to measure plasma glucose, insulin, free fatty acids, and triglycerides (at t=-45, t=15, t=150, t=330). In between scans, volunteers bed-rested and indirect calorimetry (ventilated hood) was used to determine metabolic flexibility ($\Delta\text{RER}_{t=90-\text{fasted}}$ and $\Delta\text{RER}_{t=270-t=90}$) and substrate oxidation 1.5 hours (t=90 min) and 4.5 hours (t=270 min) after consumption of the breakfast. A schematic overview of the test day is illustrated in figure 2.

Figure 2. Flowchart of the meal test (day 30). Participants were asked to consume a standardized evening meal on the evening before. Participants reported to the laboratory at 06.00AM in the fasted state. First, baseline indirect calorimetry and $^1\text{H-MRS}$ measurements were conducted. Between 07.45AM and 08.15AM, subjects consumed a high-energy breakfast. Directly after breakfast ($t=30$) as well as 2.5 ($t=150$) and 5.5 ($t=330$) hours after the meal, MRS measurements were repeated. Indirect calorimetry was measured three times using a ventilated hood system ($t=-90$, $t=90$, $t=270$). A blood samples was taken prior to each MRS measurement. IC, indirect calorimetry (ventilated hood), $^1\text{H-MRS}$, proton magnetic resonance spectroscopy.

Hyperinsulinemic-euglycemic clamp

On day 33, a one-step hyperinsulinemic-euglycemic clamp was performed to assess peripheral insulin sensitivity (19). Participants were asked to refrain from physical activity three days prior to the clamp and to consume a standardized carbohydrate rich meal the evening before the clamp. At the day of the clamp, 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- $^2\text{H}_2$]-glucose (0.04 ml/kg/min) was started to determine rates of glucose appearance (R_a), glucose disappearance (R_d), and endogenous glucose production (EGP) (20). After 2 hours ($t=120$), infusion of insulin (40 mU/m 2 /min) was started for 2.5 hours combined with simultaneous infusion of glucose (glucose 20%) to maintain euglycemia (5.0 mmol/L). During the basal period ($t=90$ -120 min) and the last 30 minutes 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. Metabolic flexibility is expressed as the insulin stimulated RER the basal RER. Carbohydrate and lipid oxidation was calculated according to Peronnet et al. (21).

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 hours and refrained from physical activity. After arrival at the laboratory, subjects rested for 30 minutes until the start of the baseline proton magnetic resonance

spectroscopy (^1H -MRS), using long echo times (TE) as previously reported (16), to quantify *in vivo* skeletal muscle acetylcarnitine. At 5:00 PM, participants were positioned in the MR scanner and baseline skeletal muscle acetylcarnitine concentrations were measured. Subsequently, a 30-minute cycling exercise at 70% of the participants predetermined maximal power output ($W_{\max}$) was performed on an ergometer in a room next to the MR scanner. Directly after the exercise, a second ^1H -MRS measurement was performed to quantify skeletal muscle acetylcarnitine concentrations.

^1H -MRS (acetylcarnitine)

In vivo skeletal muscle acetylcarnitine concentration was measured using a long-TE ^1H -MRS protocol as described previously (16). All experiments were performed on a 3T clinical MR scanner (Achieva 3T-X, Philips Healthcare, Best, The Netherlands). Participants were positioned in supine position feet first in the MR scanner and a two-element surface coil was placed around the left upper leg. The voxel of interest (40 mm x 20 mm x 60 mm) was positioned in the *m. vastus lateralis*.

The spectra were acquired with the following acquisition parameters: PRESS, TR=6000, spectral bandwidth 2 kHz 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, acetylcarnitine concentration was analyzed in spectra with TE=500 ms as the shorter echo times showed considerable lipid contamination in overweight participants. Baseline correction was performed for all acquired spectra with a custom-made MATLAB script (The Mathworks Inc.). Spectra were analyzed using the AMARES algorithm in jMRUI software (22). The creatine resonance (t-Cr) was used as internal reference and acetylcarnitine concentration was calculated assuming a creatine concentration of 30 mmol/kg ww. T_2 corrections were performed for creatine ($T_2=166$ ms) and acetylcarnitine ($T_2=262$ ms) and a correction for the dipolar coupling of creatine was applied and set at 30% of the signal as reported earlier (16).

Blood analysis

Arterialized blood samples (hotbox heated) were collected from a hand vein during the hyperinsulinemic-euglycemic clamp and were immediately centrifuged. Venous blood samples obtained during the meal test were collected identically. Plasma was frozen in liquid nitrogen and stored at -80°C until analyzed. Plasma concentrations of insulin,

glucose, free fatty acids (FFAs), and total and free glycerol were determined in duplicate. Plasma FFAs, glucose and free and total glycerol were measured using enzymatic assays automated on a Cobas Fara/Mira analyzer [glucose: hexokinase method (Roche); FFAs: Wako Nefa C test kit (Wako Chemicals); glycerol: Enzytec glycerol kit (R-biopharm)]. Free glycerol and total glycerol were used to calculate triacylglyceride concentrations. Plasma insulin concentrations were determined using an RIA (Millipore). Plasma acylcarnitine concentrations were measured by tandem mass spectrometry as previously reported (23). Acylcarnitine concentrations were determined at the start end of the intervention (day 0 and 33).

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 (24) under local anesthesia (2% Lidocaine, Accord Healthcare Limited, Harrow, United Kingdom). Muscle tissue was directly frozen in melting isopentane and stored at -80°C until further processing. Skeletal muscle acylcarnitines were analyzed as previously described using mass spectrometry (25). Glycogen concentration was determined by using a commercial glycogen assay kit (ab65620, Abcam, Cambridge, United Kingdom) according to instructions of the manufacturer.

Tracer calculations

Isotopic enrichment of plasma glucose was determined by electron ionization gas chromatography-mass spectrometry as described previously (26). Steeles single pool non-steady state equations were used to calculate glucose R_a and R_d (20). Volume of distribution was assumed to be 0.160 L/kg for glucose. Insulin-stimulated glucose disposal (ΔR_d) was computed as the difference between R_d under insulin-stimulated conditions and R_d under basal non-insulin stimulated conditions. EGP was calculated as R_a minus exogenous glucose infusion rate. Non-oxidative glucose disposal (NOGD) was calculated as R_d minus carbohydrate oxidation, derived from the indirect calorimetry measurements.

Statistics

Data are presented as means $\pm$ SEM. Statistical analysis were performed using SPSS 24.0 software (SPSS, Chicago, IL.). Results were considered to be significantly different when p value <0.05 (two-sided testing). To evaluate if the data were normally

distributed a Shapiro-Wilk normality test was performed. Statistical comparisons between the two intervention trials (carnitine and placebo) were performed using a Students paired sample t-test. Students independent sample t-tests were performed to compare NGT participants to IGT participants. 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. (27). No carry-over or period effects were found.

RESULTS

Subject characteristics

Body composition and maximal oxygen uptake (VO_{2max}) were comparable between groups. Fasting glucose levels were slightly higher in IGT participants ($p=0.034$) and, by design, glucose levels 120 minutes after an oral glucose tolerance test ($OGTT_{120}$) were higher in IGT ($p<0.01$). Also insulin levels at $t=120$ minutes 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 (supplementary table 1).

Plasma free carnitine and acetylcarnitine concentrations

No difference in plasma free carnitine (C0) concentrations were found between the placebo and carnitine group at the start of the intervention ($p=0.312$). In line, plasma acetylcarnitine concentration did not differ between the groups. 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 $\mu\text{mol/L}$, $p<0.01$ and C2 from 6.6 ± 0.4 to 8.3 ± 0.6 $\mu\text{mol/L}$, $p=0.035$), indicating compliance to the study protocol (figure 1). C3 acylcarnitine species also increased upon carnitine supplementation. No differences in other acylcarnitine species were detected upon carnitine supplementation (supplementary table 2). Carnitine levels did not change after placebo treatment (C0 from 39.4 ± 1.4 to 39.8 ± 1.3 $\mu\text{mol/L}$, $p=0.785$ and C2 from 6.0 ± 0.4 to 6.2 ± 0.3 $\mu\text{mol/L}$, $p=0.489$).

Figure 1. Plasma free carnitine concentrations (A) and plasma acetylcarnitine concentration (B). In A and B, black bars represent day 0 and white bars represent day 33 in NGT, IGT P (IGT placebo), and IGT C (IGT carnitine). Data are expressed as means $\pm$ SEM. * significantly different ($P<0.05$).

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/m ²)	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)	64.0 ± 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		
VO ₂ max (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 OGTT ₁₂₀ (mmol/L)	4.6 ± 0.3	8.2 ± 0.3*
Fasting insulin (pmol/L)	56.6 ± 9.1	80.1 ± 14.2
Insulin OGTT ₁₂₀ (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

Data are represented as mean ± SEM. * p<0.05, IGT significantly different from NGT. Wmax, maximal workload.

Metabolic flexibility and insulin sensitivity

Metabolic flexibility, expressed as the change in respiratory exchange ratio upon insulin stimulation (Δ RER), was significantly lower in IGT compared to NGT participants (figure 2A, p=0.022). Interestingly, carnitine supplementation completely restored metabolic flexibility to NGT values (figure 2A, p<0.05). Overnight fasted lipid and carbohydrate oxidation rates were not different between NGT and IGT. However, carnitine supplementation increased overnight fasted basal whole-body lipid oxidation compared to placebo (p=0.034), resulting in fat oxidation levels that were higher than in normal glucose tolerant participants (figure 2C, 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 ($p=0.055$). Despite the reduction in carbohydrate oxidation in the fasted state in IGT participants upon carnitine supplementation, non-oxidative glucose disposal in the fasted state was increased ($p<0.05$) 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. To this end, we performed a hyperinsulinemic euglycemic clamp combined with deuterated glucose tracers to allow the determination of the rate of disappearance of glucose, which mainly reflects skeletal muscle glucose uptake. Basal EGP was lower in IGT participants compared to NGT ($p<0.01$) but was not affected by carnitine supplementation ($p=0.962$). Also, insulin-stimulated suppression of EGP was not significantly different between groups ($p>0.05$). As expected, the insulin-stimulated rate of disappearance of glucose (ΔR_d) was significantly higher in NGT compared to IGT participants ($p<0.01$, table 2). Carnitine supplementation did not significantly affect insulin stimulated glucose disposal (ΔR_d : 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 2). 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.

Figure 2. Metabolic flexibility measured as the change from the fasted state to the insulin-stimulated state (ΔRER) (A), CHO oxidation (B), and lipid oxidation (C). In B and C, black bars represent the fasted state and white bars represent the insulin-stimulated state in NGT, IGT P (IGT placebo), and IGT C (IGT carnitine). Data are expressed as means $\pm$ SEM. * insulin stimulated state significantly different from fasted state, # different from insulin stimulated state NGT, ^different from fasted state NGT and IGT on placebo ($p<0.05$).

Table 2. Substrate kinetics and insulin sensitivity

	NGT (n=12)	IGT Placebo (n=11)	IGT Carnitine (n=11)
RER (arbitrary units AU)			
Basal	0.798 ± 0.008	0.791 ± 0.012	0.762 ± 0.010 ^{a,b}
Insulin-stimulated	0.899 ± 0.009	0.857 ± 0.011 ^a	0.863 ± 0.010 ^a
Δ	0.101 ± 0.012	0.066 ± 0.007 ^a	0.100 ± 0.010 ^b
CHO oxidation (μmol*kg ⁻¹ *min ⁻¹)			
Basal	6.71 ± 0.61	5.93 ± 0.71	4.01 ± 0.66 ^{a,b}
Insulin-stimulated	14.22 ± 0.78	10.77 ± 0.75	11.09 ± 0.79 ^a
Δ	7.51 ± 1.01	4.84 ± 0.48	7.08 ± 0.74 ^b
Lipid oxidation (μmol*kg ⁻¹ *min ⁻¹)			
Basal	1.10 ± 0.05	1.12 ± 0.08	1.27 ± 0.07 ^{a,b}
Insulin-stimulated	0.55 ± 0.05	0.77 ± 0.07	0.73 ± 0.06 ^a
Δ	-0.54 ± 0.07	-0.35 ± 0.04	-0.54 ± 0.05 ^b
Rd glucose (μmol*kg ⁻¹ *min ⁻¹)			
Basal	10.80 ± 0.70	7.64 ± 0.49 ^a	7.97 ± 0.80 ^a
Insulin-stimulated	36.51 ± 3.48	19.38 ± 2.02 ^a	21.29 ± 2.98 ^a
Δ	25.71 ± 3.39	11.74 ± 1.99 ^a	13.32 ± 3.08 ^a
NOGD (μmol*kg ⁻¹ *min ⁻¹)			
Basal	5.41 ± 0.70	3.09 ± 0.49 ^a	5.94 ± 0.80 ^b
Insulin-stimulated	23.16 ± 3.48	10.98 ± 2.02 ^a	9.08 ± 2.98 ^a
Δ	17.76 ± 3.39	7.89 ± 1.99 ^a	3.15 ± 3.08 ^a
Glucose (mmol/L)			
Basal	5.5 ± 0.1	5.8 ± 0.1 ^a	5.9 ± 0.1 ^a
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 ^a	110.30 ± 11.28 ^a

Data are expressed as mean ± SEM. ^a IGT placebo or IGT carnitine significantly different from NGT. ^b IGT carnitine significantly different from IGT placebo.

Table 3. Substrate kinetics and insulin sensitivity

	NGT	IGT Placebo	IGT Carnitine
RER			
T1=-90 (baseline)	0.836 ± 0.009	0.829 ± 0.012	0.795 ± 0.015 ^{a,b}
T2=90	0.860 ± 0.006	0.830 ± 0.012 ^a	0.833 ± 0.009 ^a
T3=270	0.816 ± 0.010	0.806 ± 0.016	0.796 ± 0.007
Δ T2-T1	0.022 ± 0.007	0.001 ± 0.013	0.037 ± 0.008 ^b
Δ T3-T2	-0.044 ± 0.009	-0.024 ± 0.012	-0.037 ± 0.008
CHO oxidation (μmol*kg ⁻¹ *min ⁻¹)			
T1=-90 (baseline)	9.56 ± 0.82	8.66 ± 0.86	6.86 ± 1.04 ^{#,b}
T2=90	12.50 ± 0.58	9.57 ± 0.82 ^a	9.88 ± 0.61 ^a
T3=270	8.83 ± 0.74	8.08 ± 1.21	7.25 ± 0.61
Δ T2-T1	3.74 ± 1.06	0.91 ± 0.98 [#]	3.02 ± 0.80 ^b
Δ T3-T2	-3.67 ± 0.70	-1.49 ± 0.89 [#]	-2.63 ± 0.58
Lipid oxidation (μmol*kg ⁻¹ *min ⁻¹)			
T1=-90 (baseline)	0.83 ± 0.06	0.91 ± 0.07	1.04 ± 0.10 ^{#,%}
T2=90	0.85 ± 0.05	1.01 ± 0.10	0.99 ± 0.07
T3=270	1.12 ± 0.08	1.17 ± 0.09	1.23 ± 0.05
Δ T2-T1	0.09 ± 0.08	0.10 ± 0.08	-0.05 ± 0.06 [%]
Δ T3-T2	0.26 ± 0.06	0.16 ± 0.08	0.25 ± 0.05

Data are expressed as mean ± SEM. ^a IGT placebo or IGT carnitine significantly different from NGT.

^b IGT carnitine significantly different from IGT placebo. [#]IGT placebo or IGT carnitine tending to be different from NGT. [%] IGT carnitine tending to be different from IGT placebo.

Skeletal muscle glycogen concentrations

To directly test if carnitine supplementation affected baseline muscle glycogen concentrations, we measured glycogen concentration in muscle biopsies taken before the clamp. Muscle glycogen was not different between NGT and IGT participants ($p>0.05$). Although not significant, skeletal muscle glycogen concentrations after an overnight fast tended to increase upon carnitine supplementation ($p=0.142$, figure 3). These data are in line with a higher non-oxidative glucose disposal (NOGD) in the overnight fasted state upon carnitine supplementation ($p=0.006$), suggesting that under basal conditions more glucose is shuttled towards glycogen storage. It also may explain the reduction in insulin-stimulated non-oxidative glucose disposal as basal glycogen levels are known to determine further rates of glycogen storage.

Figure 3. Skeletal muscle glycogen concentrations. Data are expressed as means $\pm$ SEM.

Figure 4. Respiratory exchange ratio (A), black bars represent $\Delta RER_{t=90-\text{basal}}$ and white bars represent $\Delta RER_{t=270-t=90}$. * significantly different from IGT on placebo, # different from NGT. CHO oxidation (B), and lipid oxidation (C). In B and C, black bars represent the fasted state, white bars represent $t=90$ min and grey bars represent $t=270$ min after the meal in NGT, IGT P (IGT placebo), and IGT C (IGT carnitine). Data are expressed as means $\pm$ SEM. * significantly different from fasted state, # different from NGT and IGT, ^different from $t=90$ min, & different from NGT, % different from $t=90$ ($P<0.05$). Values refer to twelve participants in the NGT group and nine participants in the IGT P and IGT C groups. Two IGT participants were not able to perform the meal test due to technical problems.

Metabolic flexibility assessed by a high-energy meal test

Next to the assessment of metabolic flexibility during a clamp, metabolic flexibility was also determined in a more physiological setting, in this case before and after a meal. In line with the metabolic flexibility 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 ($p < 0.05$ figure 4A). 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 ($p = 0.027$). The lower basal RER upon carnitine supplement reflected a decreased carbohydrate oxidation ($p = 0.031$, figure 4B) and a trend towards increased whole-body lipid oxidation ($p = 0.056$, figure 4C) compared to placebo. Although metabolic flexibility improved meal-induced changes in plasma glucose, insulin, triacylglycerides and free fatty acids were unaffected by carnitine supplementation (figure 5).

Figure 5. Plasma metabolites during the meal test, glucose (A), free fatty acids FFA (B), triglycerides (C), insulin (D). 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 $\pm$ 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.

Carnitine supplementation improves capacity to form acetylcarnitine in muscle

To investigate if the improved metabolic flexibility upon carnitine supplementation was due to improved skeletal muscle acetylcarnitine metabolism, we used ^1H -MRS to determine acetylcarnitine levels in the vastus lateralis muscle. This non-invasive technique allows the repeated measurement of acetylcarnitine in the same muscle group. 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 $\mu\text{mol/kgww}$ 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 as measured in biopsies taken after an overnight fast. Likewise in the muscle biopsies, no difference between the groups or between the placebo and carnitine treatment were detected (see data below). Given the hypothesized role of acetyl-carnitine 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 when compared to 7:00 AM in NGT (figure 6), suggesting that acetylcarnitine levels in skeletal muscle rise during the day. Intriguingly, this increase in skeletal muscle acetylcarnitine levels during the day (assessed in a subset of 8 participants) was markedly blunted in the IGT group (delta acetylcarnitine concentration: 0.67 ± 0.18 vs 1.51 ± 0.33 $\mu\text{mol/kgww}$ in IGT compared to NGT, $p=0.037$, figure 6), but was completely restored upon carnitine supplementation (1.57 ± 0.33 mmol/kgww). As a result, acetylcarnitine 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 acetylcarnitine levels towards values observed in NGT (1.62 ± 0.27 mmol/kgww). Finally, we also determined the maximal capacity to form acetylcarnitine. Exercise is known to lead to an increase in skeletal muscle acetylcarnitine, possibly because substrate load into the mitochondria is rapidly increased upon exercise thereby elevating acetyl-CoA levels. At high exercise intensity, this increase in acetylcarnitine reflects the capacity individuals have to produce acetylcarnitine and can be seen as a parameter for free carnitine availability. Therefore, acetylcarnitine was measured in skeletal muscle before exercise (5:00 PM measurement)

and after 30 minutes of exercise at 70% W_{max} . Acetylcarnitine levels in skeletal muscle increased in all three groups ($p < 0.05$), but post-exercise acetylcarnitine 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, figure 7), suggesting that carnitine supplementation boosted the maximal capacity to form acetylcarnitine.

Figure 6. Skeletal muscle acetylcarnitine concentrations over the day (A) and delta acetylcarnitine concentrations (B). In A, 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 $\pm$ SEM. * IGT placebo significantly different from IGT carnitine and NGT, # IGT placebo significantly different from IGT on carnitine ($p < 0.05$). Values refer to twelve participants in the NGT group and eight participants in the IGT P and IGT C groups. Two IGT participants were not able to perform the 07:00 AM measurement due to technical problems and one subject was excluded due to protocol violations.

Figure 7. Skeletal muscle acetylcarnitine concentrations in the resting state and the capacity to form acetylcarnitine with exercise in NGT, IGT P (IGT placebo), and IGT C (IGT carnitine). Data are expressed as means $\pm$ SEM. * significantly different from IGT on carnitine, # tending to be different from NGT ($p = 0.064$). Values refer to twelve participants in the NGT group and eleven participants in the IGT P and IGT C groups.

Skeletal muscle acylcarnitine profiles

A limitation of MRS is that only acetylcarnitine levels can be measured and no information is provided about other acylcarnitine species. Therefore, we examined the complete acylcarnitine profile in the muscle biopsies taken after an overnight fast after placebo or carnitine supplementation. As described above, no difference in free carnitine (C0), acetylcarnitine (C2) or other short-chain acylcarnitine concentrations were detected in muscle biopsies between NGT and IGT ($p > 0.05$) and carnitine supplementation did not affect this ($p > 0.05$). Long-chain acylcarnitines C16:2 and C20:2, however, tended to be elevated in IGT on placebo compared to NGT ($p < 0.05$), suggesting less complete β -oxidation in IGT volunteers. Interestingly, carnitine supplementation lowered long-chain C18:2, C18:1, C18 and C20:1 ($p < 0.05$) and tended to lower C16 and C20 ($p < 0.1$) acylcarnitine species (figure 8, supplementary table 3), suggesting carnitine supplementation contributed to more complete β -oxidation.

Figure 8. Skeletal muscle long-chain acylcarnitine concentrations measured in biopsies. Black bars represent the NGT group, white bars the IGT on placebo and grey bars IGT on carnitine. Data are expressed as means $\pm$ 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 ($p < 0.10$).

DISCUSSION

Animal studies have indicated that free carnitine availability in skeletal muscle may be crucial in the formation of acetylcarnitine and subsequently maintenance of metabolic flexibility as indicated by Noland et al. (4). We here aimed to investigate in humans if carnitine supplementation leads to increased acetylcarnitine formation and improves metabolic flexibility and insulin sensitivity in individuals with impaired glucose tolerance. In the current study we show that metabolic flexibility was lower in IGT compared to NGT participants and could be completely restored upon carnitine supplementation. Concomitantly, free carnitine availability in plasma increased. Likewise, the increase in *in vivo* resting skeletal muscle acetylcarnitine concentrations during the day was enhanced upon carnitine supplementation compared to placebo in impaired glucose tolerant volunteers, as well as the maximal capacity to form acetylcarnitine during exercise. These findings are in line with the notion that the capacity to form acetylcarnitine is important in mitochondrial substrate switching and may be limited by availability of free carnitine (3, 4). Interestingly, improved metabolic flexibility was not only measured during the hyperinsulinemic euglycemic clamp, but also in a more physiological setting upon consumption of an energy-rich meal. Together these data suggest that also in humans, carnitine supplementation may help to improve metabolic flexibility via increasing skeletal muscle acetylcarnitine levels.

In the current study, we determined acetylcarnitine concentrations *in vivo* using ^1H -MRS both in the morning after an overnight fast as well as in the afternoon. Interestingly, acetylcarnitine concentrations were consistently higher in the afternoon compared to the morning, suggesting that acetylcarnitine levels rise during the day. This increase in acetylcarnitine levels during the day fits with a role of muscle acetylcarnitine in facilitating metabolic flexibility, as it is known that after an overnight fast the body mainly relies on fatty acid oxidation, whereas substrate oxidation is more mixed later during the day when the body is in a fed state (1). Strikingly, this increase in acetylcarnitine over the day was substantially blunted in IGT, leading to significant differences in acetylcarnitine concentrations between the groups in the afternoon, which were not detectable in the morning. Interestingly, upon carnitine supplementation the increase of acetylcarnitine levels during the day was completely restored to NGT levels, which makes it tempting to speculate that the variation in acetylcarnitine levels over the day may be related to flexibility in substrate use.

Underlying mechanisms of improved metabolic flexibility upon carnitine supplementation are yet unknown, but indirect calorimetry showed that especially basal lipid oxidation was increased, thereby sparing glycogen stores. In line with the latter, muscle glycogen concentrations and non-oxidative glucose disposal rate in the basal, fasted state were enhanced upon carnitine supplementation. With respect to the elevated fat oxidation, accumulation of long-chain acylcarnitines in skeletal muscle has previously been reported in situations when high fat availability drives fat oxidation. Under such circumstances, fatty acid availability surpasses fat oxidative capacity, resulting in incomplete fat oxidation (4, 6, 7). Contrary to such availability-driven increases in fat oxidation, the increased fat oxidation in the current study was accompanied by decreased levels of skeletal muscle long-chain acylcarnitines. Thus, this suggests that in the current study, carnitine supplementation resulted in pronounced improvements in fat oxidative capacity, stimulating complete fat oxidation. In accordance, findings of higher fat oxidative capacity and lower skeletal muscle long-chain acylcarnitines were found when wild type mice were compared with SIRT3 knock-out mice (28-31). SIRT3 is a deacetylase located in the mitochondrial matrix and is known for its function in regulating acetylation of multiple metabolic proteins and enzymes. The downregulation of SIRT3 results in increased lysine acetylation of mitochondrial proteins/enzymes, which may give rise to incomplete fat oxidation and hence the accumulation of long-chain acylcarnitines (28-32). Lysine acetylation of mitochondrial proteins is thought to be the resultant of the mass action of excessive carbon load, such as during exercise or over-feeding, as accumulating acetyl-CoA levels are expanding the acetyl donor pool (33-35). Davies et al. showed that high acetyl-CoA concentrations can indeed induce lysine acetylation in mice. Interestingly with respect to the current study, this effect can be blocked by supplementing the mice with carnitine (33). Although we did not investigate protein acetylation, it is well known that important regulatory enzymes of the β -oxidation and glycogen synthesis pathway can be acetylated (33). Therefore, it is tempting to speculate that the low metabolic flexibility in pre-diabetic volunteers is due to increased lysine acetylation and that carnitine supplementation may have reduced lysine acetylation by stimulating the formation of acetylcarnitine and thereby keeping intramitochondrial acetyl-CoA levels low. Such changes in acetyl-donor pool and consequently reduced acetylation could explain the beneficial effects observed on metabolic flexibility and fat oxidation capacity in pre-diabetic patients. Future studies are necessary to investigate if acetylation might underlie the beneficial effects on metabolic flexibility in pre-diabetic patients.

Despite the beneficial effects of carnitine supplementation on skeletal muscle acetylcarnitine 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. (36) and Galloway et al. (37) 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.

In conclusion, we here show that carnitine supplementation has very pronounced effects of on metabolic flexibility in impaired glucose tolerant volunteers, and in fact can completely restore metabolic flexibility. Carnitine supplementation enhanced the increase in acetylcarnitine concentration in resting muscle over the day as well as the capacity to form acetylcarnitine with exercise. These changes in acetylcarnitine 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.

REFERENCES

1. Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes*. 2000;49(5):677-83.
2. Faerch K, Vaag A. Metabolic inflexibility is a common feature of impaired fasting glycaemia and impaired glucose tolerance. *Acta diabetologica*. 2011;48(4):349-53.
3. Muoio DM, Noland RC, Kovalik JP, Seiler SE, Davies MN, DeBalsi KL, et al. Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. *Cell metabolism*. 2012;15(5):764-77.
4. Noland RC, Koves TR, Seiler SE, Lum H, Lust RM, Ilkayeva O, et al. Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control. *The Journal of biological chemistry*. 2009;284(34):22840-52.
5. Stephens FB, Constantin-Teodosiu D, Greenhaff PL. New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *The Journal of physiology*. 2007;581(Pt 2):431-44.
6. Muoio DM. Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. *Cell*. 2014;159(6):1253-62.
7. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell metabolism*. 2008;7(1):45-56.
8. Liepinsh E, Makrecka-Kuka M, Makarova E, Volska K, Svalbe B, Sevostjanovs E, et al. Decreased acylcarnitine content improves insulin sensitivity in experimental mice models of insulin resistance. *Pharmacological research*. 2016;113(Pt B):788-95.
9. Power RA, Hulver MW, Zhang JY, Dubois J, Marchand RM, Ilkayeva O, et al. Carnitine revisited: potential use as adjunctive treatment in diabetes. *Diabetologia*. 2007;50(4):824-32.
10. Ringseis R, Keller J, Eder K. Role of carnitine in the regulation of glucose homeostasis and insulin sensitivity: evidence from in vivo and in vitro studies with carnitine supplementation and carnitine deficiency. *European journal of nutrition*. 2012;51(1):1-18.
11. De Palo E, Gatti R, Sicolo N, Padovan D, Vettor R, Federspil G. Plasma and urine free L-carnitine in human diabetes mellitus. *Acta diabetologica latina*. 1981;18(1):91-5.

12. Molfino A, Cascino A, Conte C, Ramaccini C, Rossi Fanelli F, Laviano A. Caloric restriction and L-carnitine administration improves insulin sensitivity in patients with impaired glucose metabolism. *JPEN Journal of parenteral and enteral nutrition*. 2010;34(3):295-9.
13. Malaguarnera M, Gargante MP, Russo C, Antic T, Vacante M, Malaguarnera M, et al. L-carnitine supplementation to diet: a new tool in treatment of nonalcoholic steatohepatitis—a randomized and controlled clinical trial. *The American journal of gastroenterology*. 2010;105(6):1338-45.
14. Rahbar AR, Shakerhosseini R, Saadat N, Taleban F, Pordal A, Gollestan B. Effect of L-carnitine on plasma glycemic and lipidemic profile in patients with type II diabetes mellitus. *European journal of clinical nutrition*. 2005;59(4):592-6.
15. Ruggerenti P, Cattaneo D, Loriga G, Ledda F, Motterlini N, Gherardi G, et al. Ameliorating hypertension and insulin resistance in subjects at increased cardiovascular risk: effects of acetyl-L-carnitine therapy. *Hypertension (Dallas, Tex : 1979)*. 2009;54(3):567-74.
16. Lindeboom L, Nabuurs CI, Hoeks J, Brouwers B, Phielix E, Kooi ME, et al. Long-echo time MR spectroscopy for skeletal muscle acetylcarnitine detection. *The Journal of clinical investigation*. 2014;124(11):4915-25.
17. WHO Expert Committee on Diabetes Mellitus: second report. *World Health Organization technical report series*. 1980;646:1-80.
18. Cunningham JJ. A reanalysis of the factors influencing basal metabolic rate in normal adults. *The American journal of clinical nutrition*. 1980;33(11):2372-4.
19. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *The American journal of physiology*. 1979;237(3):E214-23.
20. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. *Annals of the New York Academy of Sciences*. 1959;82:420-30.
21. Peronnet F, Massicotte D. Table of nonprotein respiratory quotient: an update. *Can J Sport Sci*. 1991;16(1):23-9.
22. Vanhamme L, van den Boogaart A, Van Huffel S. Improved method for accurate and efficient quantification of MRS data with use of prior knowledge. *Journal of magnetic resonance (San Diego, Calif : 1997)*. 1997;129(1):35-43.
23. Vreken P, van Lint AE, Bootsma AH, Overmars H, Wanders RJ, van Gennip AH. Quantitative plasma acylcarnitine analysis using electrospray tandem mass spectrometry for the diagnosis of organic acidaemias and fatty acid oxidation defects. *Journal of inherited metabolic disease*. 1999;22(3):302-6.

24. Bergstrom J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance. *Acta physiologica Scandinavica*. 1967;71(2):140-50.
25. van Vlies N, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, et al. Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. *The Biochemical journal*. 2005;387(Pt 1):185-93.
26. Ackermans MT, Pereira Arias AM, Bisschop PH, Endert E, Sauerwein HP, Romijn JA. The quantification of gluconeogenesis in healthy men by (2)H₂O and [2-(13)C]glycerol yields different results: rates of gluconeogenesis in healthy men measured with (2)H₂O are higher than those measured with [2-(13)C]glycerol. *The Journal of clinical endocrinology and metabolism*. 2001;86(5):2220-6.
27. S P. *Clinical Trials: A Practical Approach*. Chichester: John Wiley and Sons; 1987.
28. Hirschey MD, Shimazu T, Goetzman E, Jing E, Schwer B, Lombard DB, et al. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. *Nature*. 2010;464(7285):121-5.
29. Jing E, Emanuelli B, Hirschey MD, Boucher J, Lee KY, Lombard D, et al. Sirtuin-3 (Sirt3) regulates skeletal muscle metabolism and insulin signaling via altered mitochondrial oxidation and reactive oxygen species production. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108(35):14608-13.
30. Lantier L, Williams AS, Williams IM, Yang KK, Bracy DP, Goelzer M, et al. SIRT3 Is Crucial for Maintaining Skeletal Muscle Insulin Action and Protects Against Severe Insulin Resistance in High-Fat-Fed Mice. *Diabetes*. 2015;64(9):3081-92.
31. Hirschey MD, Shimazu T, Jing E, Grueter CA, Collins AM, Aouizerat B, et al. SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome. *Molecular cell*. 2011;44(2):177-90.
32. Dittenhafer-Reed KE, Richards AL, Fan J, Smallegan MJ, Fotuhi Siahpirani A, Kemmerer ZA, et al. SIRT3 mediates multi-tissue coupling for metabolic fuel switching. *Cell metabolism*. 2015;21(4):637-46.
33. Davies MN, Kjalarsdottir L, Thompson JW, Dubois LG, Stevens RD, Ilkayeva OR, et al. The Acetyl Group Buffering Action of Carnitine Acetyltransferase Offsets Macronutrient-Induced Lysine Acetylation of Mitochondrial Proteins. *Cell reports*. 2016;14(2):243-54.
34. Ghanta S, Grossmann RE, Brenner C. Mitochondrial protein acetylation as a cell-intrinsic, evolutionary driver of fat storage: chemical and metabolic logic of acetyl-lysine modifications. *Critical reviews in biochemistry and molecular biology*. 2013;48(6):561-74.

35. Wagner GR, Payne RM. Widespread and enzyme-independent Nepsilon-acetylation and Nepsilon-succinylation of proteins in the chemical conditions of the mitochondrial matrix. *The Journal of biological chemistry*. 2013;288(40):29036-45.
36. Gonzalez-Ortiz M, Hernandez-Gonzalez SO, Hernandez-Salazar E, Martinez-Abundis E. Effect of oral L-carnitine administration on insulin sensitivity and lipid profile in type 2 diabetes mellitus patients. *Annals of nutrition & metabolism*. 2008;52(4):335-8.
37. Galloway SD, Craig TP, Cleland SJ. Effects of oral L-carnitine supplementation on insulin sensitivity indices in response to glucose feeding in lean and overweight/obese males. *Amino acids*. 2011;41(2):507-15.

SUPPLEMENTARY DATA

Supplementary Table. 1. Plasma acylcarnitine 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	p=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 ± 0.1	
Insulin (pmol/L)			
Day 0	68.8 ± 9.0	78.6 ± 11.7	p=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	p=0.921
Day 33	5.5 ± 0.1	5.6 ± 0.1	
Blood lipid profile			
Total cholesterol (mmol/L)			
Day 0	5.7 ± 0.3	5.9 ± 0.2	p=0.521
Day 7	5.7 ± 0.3	5.8 ± 0.2	
Day 14	5.6 ± 0.3	6.0 ± 0.3	
Day 21	5.5 ± 0.2	5.7 ± 0.2	
Day 28	5.6 ± 0.3	5.1 ± 0.3	
Day 33	5.4 ± 0.2	5.5 ± 0.3	

HDL cholesterol (mmol/L)			
Day 0	1.4 ± 0.1	1.4 ± 0.1	p=0.391
Day 7	1.4 ± 0.1	1.4 ± 0.2	
Day 14	1.2 ± 0.1	1.4 ± 0.2	
Day 21	1.4 ± 0.1	1.2 ± 0.1	
Day 28	1.6 ± 0.2	1.1 ± 0.1	
Day 33	1.3 ± 0.1	1.3 ± 0.1	
LDL cholesterol (mmol/L)			
Day 0	3.4 ± 0.3	3.6 ± 0.2	p=0.620
Day 7	3.5 ± 0.3	3.6 ± 0.3	
Day 14	3.5 ± 0.3	3.5 ± 0.2	
Day 21	3.3 ± 0.3	3.3 ± 0.2	
Day 28	3.3 ± 0.1	2.9 ± 0.2	
Day 33	3.2 ± 0.2	3.2 ± 0.2	
Triglycerides (mmol/L)			
Day 0	2.1 ± 0.3	2.6 ± 0.5	p=0.512
Day 7	2.1 ± 0.3	2.6 ± 0.5	
Day 14	2.5 ± 0.5	3.6 ± 1.5	
Day 21	2.9 ± 0.5	3.0 ± 0.4	
Day 28	2.6 ± 0.3	2.2 ± 0.4	
Day 33	2.0 ± 0.2	2.1 ± 0.3	
Liver function			
ASAT (U/L)			
Day 0	27.1 ± 2.3	25.2 ± 2.0	p=0.678
Day 33	23.7 ± 1.5	23.6 ± 3.4	
ALAT (U/L)			
Day 0	28.1 ± 3.6	29.8 ± 3.4	p=0.758
Day 33	25.6 ± 2.7	32.3 ± 4.5	
Gamma-GT (U/L)			
Day 0	39.2 ± 8.2	39.4 ± 6.3	p=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 two-way repeated measures ANOVA. Plasma values are obtained after an overnight fast.

Supplementary Table. 2. Plasma acylcarnitine before and after 30 days of carnitine and placebo

	NGT	IGT placebo	IGT carnitine	IGT placebo	IGT carnitine
	Day 0	Day 0	Day 0	Day 33	Day 33
C0	42.48 ± 1.19	39.43 ± 1.44	40.77 ± 1.57	39.75 ± 1.31	50.52 ± 1.66 ^{a,b,c}
C2	5.52 ± 0.35	5.95 ± 0.36	6.63 ± 0.44	6.24 ± 0.35	8.28 ± 0.61 ^{a,b,c}
C3	0.44 ± 0.02	0.42 ± 0.04	0.45 ± 0.05	0.47 ± 0.03	0.55 ± 0.04 ^{a,b,c}
C4	0.35 ± 0.01	0.31 ± 0.01	0.33 ± 0.03	0.32 ± 0.02	0.34 ± 0.02 ^c
C5:1	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00 ^a
C5	0.11 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.01
C4-3OH	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
C6	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
C5OH	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C8	0.21 ± 0.03	0.23 ± 0.02	0.25 ± 0.03	0.20 ± 0.02	0.23 ± 0.03
C3 DC	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
C10:1	0.16 ± 0.02	0.20 ± 0.02	0.22 ± 0.03	0.17 ± 0.02	0.18 ± 0.02
C10	0.25 ± 0.03	0.28 ± 0.03	0.31 ± 0.04	0.24 ± 0.03	0.27 ± 0.03
C4 DC	0.02 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.01 ^c
C5 DC	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.00	0.06 ± 0.01
C12:1	0.09 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.09 ± 0.01	0.11 ± 0.01
C12	0.09 ± 0.01	0.12 ± 0.01	0.13 ± 0.02 ^d	0.10 ± 0.01	0.11 ± 0.01
C6 DC	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C12:1OH	0.03 ± 0.01	0.07 ± 0.04	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
C12OH	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C14:2	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.04 ± 0.01
C14:1	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
C14	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
C8 DC	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00 ^b
C14:1OH	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
C14OH	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C16:1	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
C16	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
C10 DC	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C16:1OH	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C16OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18:2	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
C18:1	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
C18	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
C18:2OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18:1OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data are expressed as mean ± SEM. ^a significantly different from day 0, ^b different from IGT on placebo at day 33, ^c significantly different from NGT day 33, ^d different from NGT day 0.

Supplementary Table. 3. Skeletal muscle acylcarnitine concentration (in biopsies) before and after 30 days of carnitine and placebo

	NGT	IGT placebo	IGT carnitine
	Day 33	Day 33	Day 33
C0	6820.44 ± 838.28	7647.93 ± 495.58	8490.26 ± 933.94
C2	644.88 ± 80.30	731.41 ± 113.50	885.53 ± 175.97
C3	10.96 ± 1.39	12.73 ± 1.18	12.91 ± 1.34
C4	9.41 ± 1.37	10.59 ± 2.65	7.46 ± 1.31
C5:1	0.41 ± 0.09	0.73 ± 0.13	0.80 ± 0.22
C5	3.22 ± 0.62	4.95 ± 1.35	4.94 ± 1.15
C4-3OH	2.59 ± 0.41	4.55 ± 1.35	4.62 ± 1.25
C6	5.75 ± 1.55	7.54 ± 2.54	3.79 ± 1.36
C5-OH	1.03 ± 0.24	1.51 ± 0.29	1.48 ± 0.32
C8:1	1.03 ± 0.17	1.26 ± 0.19	0.78 ± 0.10 ^a
C8	3.24 ± 1.08	3.59 ± 1.22	1.82 ± 0.51
C3DC	0.71 ± 0.45	0.42 ± 0.15	0.72 ± 0.18
C4DC	1.77 ± 0.60	1.42 ± 0.20	1.48 ± 0.20
C10	1.78 ± 0.65	2.18 ± 0.74	1.30 ± 0.49
C5DC	1.18 ± 0.78	0.43 ± 0.12	0.52 ± 0.15
C12:1	0.42 ± 0.11	0.78 ± 0.27	0.50 ± 0.21
C12	1.62 ± 0.46	3.38 ± 1.22	2.22 ± 1.11
C8:1DC or C12:1-OH	0.12 ± 0.02	0.19 ± 0.05	0.21 ± 0.05
C8DC or C12-OH	0.13 ± 0.02	0.17 ± 0.04	0.17 ± 0.03
C14:2	0.88 ± 0.21	1.90 ± 0.66	1.33 ± 0.77
C14:1	3.17 ± 0.88	8.62 ± 3.65	5.73 ± 3.93
C14	3.20 ± 0.91	8.87 ± 3.40	5.38 ± 3.03
C10:1DC or C14:1-OH	0.32 ± 0.05	0.62 ± 0.14 ^c	0.48 ± 0.13
C16:2	0.61 ± 0.17	1.65 ± 0.57 ^d	0.98 ± 0.62
C16:1	3.21 ± 0.90	9.16 ± 3.59	5.29 ± 3.31
C16	9.15 ± 2.24	18.58 ± 5.36	12.84 ± 3.63 ^b
C12:1DC or C16:1-OH	0.31 ± 0.04	0.59 ± 0.16 ^d	0.43 ± 0.11
C18:2	3.22 ± 0.85	5.29 ± 1.35	3.50 ± 0.84 ^a

C18:1	10.72 ± 2.66	20.42 ± 6.24	12.69 ± 3.95 ^a
C18	4.02 ± 0.88	6.95 ± 1.97	5.31 ± 1.49 ^a
C20:2	0.06 ± 0.01	0.11 ± 0.02 ^d	0.08 ± 0.02
C20:1	0.19 ± 0.04	0.34 ± 0.10	0.22 ± 0.06 ^a
C20	0.07 ± 0.02	0.12 ± 0.04	0.11 ± 0.03 ^b

Data are expressed as mean ± SEM. ^a significantly different from IGT placebo, ^b tending towards a difference from IGT on placebo, ^c significantly different from NGT, ^d tending towards a difference from NGT.

Chapter 5

Longitudinal relaxation time editing for acetylcarnitine detection with ^1H -MRS

Lucas Lindeboom

Yvonne M.H. Bruls

Petronella A. van Ewijk

Matthijs K.C. Hesselink

Joachim E. Wildberger¹

Patrick Schrauwen

Vera B. Schrauwen-Hinderling

Published in Magnetic Resonance in Medicine 2016; 77(2):505-510

ABSTRACT

Purpose: Acetylcarnitine formation is suggested to be crucial in sustaining metabolic flexibility and glucose homeostasis. Recently, we introduced a method to detect acetylcarnitine *in vivo* with long TE ^1H -MRS. Differences in T_1 relaxation time between lipids and acetylcarnitine can be exploited for additional lipid suppression in subjects with high myocellular lipid levels.

Methods: Acquisition of spectra with an inversion recovery sequence was alternated with standard signal acquisition, in order to suppress short T_1 metabolite signals. A proof of principle experiment was conducted in a lean subject and the new approach was subsequently tested in four overweight/obese subjects.

Results: Using the new T_1 editing approach, lipid signals in spectra of skeletal muscle can be (additionally) suppressed by a factor of 10 using a TI of 900 ms. Combination of the long TE protocol with the T_1 editing resulted in a well-resolved acetylcarnitine peak in the obese subjects.

Conclusion: The T_1 editing approach suppresses short T_1 metabolites and offers a new contrast in ^1H -MRS. The approach should be used in combination with a long TE in subjects with high lipid contamination for accurate quantification of the acetylcarnitine concentration.

Key words: acetylcarnitine, T_1 , T_2 , inversion recovery, ^1H -MRS

INTRODUCTION

It has recently been proposed that the formation of acetylcarnitine is essential in maintaining metabolic flexibility and glucose homeostasis [1-3]. While high levels of acetyl-CoA are known to inhibit pyruvate dehydrogenase (PDH) complex activity, acetylcarnitine can be formed from excess acetyl-CoA, in a reaction catalyzed by the enzyme carnitine acetyltransferase (CrAT). As such, the formation of acetylcarnitine can be viewed as a mitochondrial rescue mechanism to maintain low acetyl-CoA concentrations and to sustain aerobic pyruvate oxidation.

While research on acetylcarnitine has long been limited to biochemical analysis in muscle biopsies, it has been shown that exercise-induced acetylcarnitine production can be detected with short echo time (TE) proton magnetic resonance spectroscopy (^1H -MRS), by analyzing pre- and post-exercise difference spectra [4]. Recently, we showed that long TE spectroscopy promotes the detectability and hence the quantification of the acetylcarnitine peak at 2.13 ppm due to TE-induced suppression of overlapping lipid resonances, thereby enabling acetylcarnitine detection even in the absence of (exercise) interventions [5].

There is a relatively large difference in transversal relaxation time (T_2) between acetylcarnitine and lipid resonances, causing the lipid signal to decay rapidly with increasing TE. Increased suppression of the lipid signal can be achieved by increasing TE, but this goes at the expense of a lower signal-to-noise-ratio of the acetylcarnitine signal. With an echo time of 350 ms, acetylcarnitine is generally well detectable in a large (48 mL) voxel in the upper leg [5]. In obese subjects, with high myocellular lipid levels, a residual lipid signal can however still result in considerable contamination of the acetylcarnitine resonance. This interferes with accurate quantification of the acetylcarnitine concentration in subjects with high lipid content in muscle, even more so, as these subjects usually show low acetylcarnitine concentrations. Alternative sources of contrast might help to improve the suppression of lipid signals in these cases.

An obvious alternative to T_2 relaxation based contrast is the use of spin lattice relaxation time (T_1) weighing. It is known that the spin-lattice relaxation time (T_1) of lipids is relatively short [6, 7] when compared to other metabolites, such as total creatine (t-Cr) [6] but also acetylcarnitine. The precise T_1 of acetylcarnitine has not

been reported, but we previously estimated the T_1 of acetylcarnitine to be 2000 ms at 3T. This is also why we used a relatively long TR of 6000 ms [5] in our previous report, which was required to achieve sufficient SNR.

T_1 contrast is traditionally achieved by using short TR. As short TR results in suppression of signals with a long T_1 relaxation time (like acetylcarnitine), it is key to reverse this traditional T_1 contrast for acetylcarnitine detection and for the concomitant suppression of the lipid resonances. This can be realized by alternating standard signal acquisition with an inversion recovery sequence with an intermediate inversion time (TI). Subtraction of both signals will result in destruction of short T_1 signals, while metabolites with a long T_1 will be retained. We here explored the use of this approach for the detection of acetylcarnitine in skeletal muscle *in vivo*.

METHODS

Methodology

The approach used in this study is schematically depicted in figure 1. Essentially, in the first acquisition an inversion recovery sequence is applied, using an adiabatic hyperbolic secant pulse with a bandwidth of 5000 Hz to invert all spins and a Point Resolved Spectroscopy (PRESS) [8] sequence for volume selection. In a second acquisition, the resonance frequency of the adiabatic inversion pulse is set far off resonance (+50 kHz), leaving the magnetization on resonance unaffected and effectively reducing the sequence to a normal PRESS scheme.

We subtracted the non-inverted spectrum from the spectrum acquired with the inversion pulse, by alternating the phase of the receiver. As metabolites with a short T_1 relaxation time will recover faster to equilibrium during the first acquisition, their contribution to the overall signal is cancelled due to the subtraction scheme. Signal yield will be highest for metabolites with a long T_1 . As this approach is based on two separate acquisitions, overall signal intensity will be lower when compared to a non-edited acquisition within the same time frame.

In this setting, optimal contrast between two metabolites is dependent on their respective T_1 relaxation times. To illustrate this, we have plotted the relative signal intensity (as $\%M_0$) as a function of the T_1 relaxation time of a metabolite for three different TIs ($TI = 300, 900$ or 1500 ms, see figure 2). The essence of our approach, i.e. suppression of short T_1 metabolites, is observable directly in this plot. Signal intensity increases with increasing T_1 . In the present case, we aim at near complete suppression of the lipid signals in the region of 2.2-2.4 ppm. These lipid resonances have T_1 relaxation rates between 200 and 450 ms [7]. For a TI of 900 ms, at least 90% lipid suppression in this region is achieved, which comes with an approximate 40% signal loss for the acetylcarnitine resonance (or any resonance with a T_1 of 2000 ms). Increasing TI will lead to an improved suppression of short T_1 metabolites, but this also comes with a decreased signal intensity of the signal of interest.

Figure 1. Schematic representation of the protocol to achieve T_1 editing. Signal acquisition with an inversion recovery sequence, using PRESS localization, is alternated with regular signal acquisition by setting the resonance frequency of the second adiabatic inversion pulse far off resonance (+50 kHz). The phase of the receiver is alternated concomitantly to suppress short T_1 metabolites (in red). Long T_1 metabolites are not relaxed to equilibrium when using intermediate TI times, leading to incomplete subtraction of these metabolites and thus observable signals.

Figure 2. Calculated relative signal intensity with the T_1 editing approach as a function of T_1 relaxation time, for three different TI times. Signal intensity is given relative to equilibrium magnetization (M_0). The lipid resonances are characterized by T_1 relaxation times between 200 and 450 ms (gray bar), while we estimated the acetylcarnitine T_1 to be 2000 ms (dotted vertical line). Relative signal intensity is plotted for a TI of 300, 900 and 1500 ms. To ensure a suppression of the lipid signals by a factor of 10, a TI of 900 ms was used in the *in vivo* protocol. Increasing TI will lead to increased suppression of short T_1 metabolites, but decreased relative acetylcarnitine signal intensity. Coming with the increased lipid suppression at a TI of 900 ms, the signal intensity of the acetylcarnitine will decay to be approximately 65% of M_0 .

Set-up and subjects

To test the performance of this novel approach, measurements were performed in a total of four subjects. In first instance, the sequence was evaluated in one a healthy subject (female, age 30 and BMI 18 kg/m²), in whom acetylcarnitine concentration was relatively high and lipid content low. Next, the added value of the novel approach above the earlier reported long TE spectroscopy was tested in four overweight/obese subject (male subjects, age 68 ± 3 years and BMI 29 ± 3 kg/m²) with strong lipid signals masking the small acetylcarnitine peak. Experiments were approved by the institutional medical ethics committee and written informed consent was obtained from the subjects prior to these experiments.

MRS acquisition protocol

All experiments were performed on a 3T clinical MR system (Achieva 3T-X, Philips

Healthcare, Best, The Netherlands) using a two-element flexible surface receive coil. Subjects were positioned supine and feet first in the magnet bore with the right foot constrained by two sandbags. The coil was placed over the *vastus lateralis* muscle. T_2 -weighted turbo spin echo images were acquired, consisting of three transversal slices and FOV= 250 x 210 mm, slice thickness= 0.9 mm, TR/TE= 2000/100 ms and turbo factor 20.

All spectra were acquired with a TR of 6000 ms. A voxel of 40 mm x 20 mm x 60 mm was positioned in the *vastus lateralis* muscle. Outer volume suppression using three rest slabs was applied, to eliminate residual signals of subcutaneous adipose tissue. An example of voxel placement in the *vastus lateralis* muscle in one of the obese subjects is shown in figure 4A. Spectral bandwidth was 2 kHz, number of acquired data points 2048, number of averages (NSA) 20 and a 4 step phase cycling was applied. Shimming was performed with FASTMAP-based shimming [9].

As proof of principle in the lean subject, a short TE (40 ms) PRESS spectrum was acquired and compared with acquisition with the T_1 editing approach with a TI of 900 ms and identical TE. Subsequently, a long TE (350 ms) PRESS spectrum without T_1 editing was compared with the T_1 editing approach using an intermediate and long TE (150 ms and 350 ms respectively). As water has a T_1 of approximately 1400 ms [6], we implemented water suppression by selective excitations and crusher gradients just prior to the PRESS excitation pulse. The flip angle of the selective excitation pulse was empirically set at 110 degrees.

In one of the obese subjects, also spectra with short TE, with and without T_1 editing, were acquired for comparison. In all four obese subjects, we compared the long TE protocol with and without T_1 editing (using TE of 150 and 350 ms). Additionally, in a separate experiment in one of the obese subjects we intentionally placed the voxel partially (approximately 10% of the total voxel volume) in the subcutaneous adipose tissue layer to maximize lipid contamination. We here tested the T_1 editing approach with a TE of 350 ms and compared this with a long TE spectrum alone. We here omitted the rest slabs for outer volume suppression.

RESULTS

Proof of principle in lean subject

Using the T_1 editing approach we were able to suppress the lipid signals in the region from 0.9 to 2.5 ppm in the short TE spectrum of the lean subject, as is visible in figure 3A. Upon application of the T_1 editing approach, the acetylcarnitine peak at 2.13 ppm is no longer masked by lipids and appears as a single peak.

A comparison between the previously described long TE protocol with and without T_1 editing indicates that a TI of 900 ms in combination with a TE of 150 ms results in similar spectra, in terms of lipid suppression and acetylcarnitine peak intensity, as illustrated in figure 3B. The combination of the long TE protocol and T_1 editing shows the additive effect of the two approaches on lipid suppression. This results in a spectrum with the lowest remaining lipid signals, which comes with signal losses of approximately 50% on the t-Cr (at 3.03 ppm) and 40% on the acetylcarnitine peak.

Application in overweight/obese subjects

In figure 4B, the short TE spectra with and without T_1 editing are shown for one of the obese subjects. In the short TE spectrum alone, large lipid signals are visible. When zooming in on the acetylcarnitine region it is clear that these lipid signals are significantly reduced with the T_1 editing approach. However, residual lipid signals that are still present make it difficult to accurately phase the spectrum. As depicted for the same obese subject in figure 4C, we found that for all four obese subjects, both the long TE protocol and T_1 editing approach with (intermediate) TE of 150 ms, showed residual lipid peaks overlapping with the acetylcarnitine resonance, thereby hindering accurate quantification of this peak. Combination of the long TE protocol with the T_1 editing approach with a TI of 900 ms, resulted in enhanced suppression of lipid signals, which in turn results in a well-resolved acetylcarnitine peak in all subjects. In analogy with the lean subject, we estimated that the T_1 editing approach comes with signal losses of approximately 50% for the t-Cr (at 3.03 ppm) and 40% for the acetylcarnitine (when assuming equal TE).

Figure 3. Proof of principle experiment in a lean subject. Spectra are acquired from the vastus lateralis muscle in a lean subject. Acquisition of a regular PRESS spectrum with TE=40 ms is compared with the T₁ editing approach with TI/TE=900/40 ms (panel A). Lipid signals are suppressed with T₁ editing. The acetylcarnitine peak at 2.13 ppm is accentuated in the grey box. In panel B, the long TE protocol is compared with the T₁ editing approach in the same subject. The T₁ editing approach (TI=900 ms) with a TE of 150 ms leads to comparable acetylcarnitine and lipid peak intensity, when compared to the long TE (350 ms) protocol. Contrast between EMCL and IMCL is slightly different, suggesting shorter T₁ of the IMCL signal. Combination of the long TE protocol with the T₁ editing approach leads to almost complete suppression of the lipid signals.

Figure 4. Performance of the T_1 editing approach in an obese subject. In panel A the voxel placement in the vastus lateralis muscle is shown. Rest slabs for outer volume suppression are shown in blue. The chemical shift dependent voxel locations are shown for the lipid resonance at 1.2 ppm (yellow box) and the t-Cr resonance at 3.03 ppm (white box). In panel B the results are depicted for short TE with and without T_1 editing (TE=40 vs. TI/TE=900/40 ms). A closer look on the acetylcarnitine region shows that residual lipid signals are still present with short TE + T_1 editing. In panel C, T_1 editing is combined with longer TE. The acetylcarnitine region of the spectrum is shown in more detail on the right part of figure. A TE of 150 ms leads to insufficient suppression of the lipid signals. Identification and quantification of the acetylcarnitine peak is also hampered in the long TE protocol alone (TE=350 ms). A combination of the long TE protocol and T_1 editing with TI 900 ms, leads to a well-resolved and well-defined acetylcarnitine peak. In panels B and C, the grey box is used to accentuate the acetylcarnitine peaks at 2.13 ppm.

Suppression of adipose tissue contamination

We have shown the spectra of the separate experiment, where we intentionally placed a part of the voxel in the subcutaneous adipose tissue, in figure 5. In the long TE spectrum the lipid signals from the adipose tissue clearly contaminate the acetylcarnitine signal, which makes the peak unobservable. The combination of the long TE protocol and the T_1 editing approach however result in complete suppression of the adipose tissue lipid signals, thereby uncovering the acetylcarnitine peak. Signal intensity of the acetylcarnitine peak was comparable with the signal intensity for the acetylcarnitine peak when the voxel was completely positioned inside the muscle tissue, which illustrates further that adipose tissue signals were completely suppressed in the acetylcarnitine region.

Figure 5. Suppression of subcutaneous adipose tissue contamination with T_1 editing. Intentional placement of part of the voxel in the subcutaneous adipose tissue around the vastus lateralis muscle, leads to large lipid signals, even with $TE=350$ ms. The acetylcarnitine peak cannot be resolved in this case. Lipid signals are suppressed by the use of the T_1 editing approach ($TI=900$ ms), as is shown in more detail in the zoom view of the region of the acetylcarnitine resonance (1.5–3 ppm region).

DISCUSSION AND CONCLUSIONS

We have shown here that differences in T_1 relaxation times of the overlapping resonances of lipids and acetylcarnitine, offer an alternative contrast mechanism to the previously described T_2 induced contrast [5] with long TE. Signal suppression of short T_1 metabolites, e.g. lipids, can be achieved by subtraction of signals from an inversion recovery of intermediate TI times (in the order of 1000 ms) from signals from regular signal acquisition. While this approach offers T_1 contrast in itself, combination of the long TE protocol with the T_1 editing approach offers improved lipid suppression and thus enhanced acetylcarnitine visibility and more accurate quantification in subjects with (very) high extra- and intramyocellular lipid signals.

The detection of acetylcarnitine *in vivo*, with ^1H -MRS was first shown by subtraction of pre- and post-exercise spectra [4], which limited its use to studies in which an exercise intervention was performed. Recently, it was suggested that acetylcarnitine formation may play a crucial role in the capability of switching between basal fat oxidation and insulin-stimulated glucose oxidation in muscle (termed metabolic flexibility). Furthermore, the capacity to form acetylcarnitine might be hampered in insulin resistant subjects [1, 2, 5]. To understand the role of acetylcarnitine in more detail, it is crucial to develop MR acquisition strategies that enable detection of this metabolite at rest, without the need for an (exercise) intervention. The relatively short T_2 of lipids enables to suppress these signals by prolonging the TE in a regular PRESS sequence [5]. For a TE of 350 ms one can calculate that approximately 98% of the lipid signal is suppressed at 3T, which is sufficient for a large subset of subjects. For obese subjects, with high myocellular lipid content, the suppression might however not suffice for accurate quantification of the acetylcarnitine signal. Augmenting lipid suppression by increasing TE above 350 ms is possible, but will unavoidably also lead to unwanted loss of acetylcarnitine signal and hence long acquisition times.

In a strive to optimize acetylcarnitine quantification for subjects with high myocellular lipid content, we estimated that differences in relative T_1 relaxation times of lipids and acetylcarnitine are even more pronounced than differences in T_2 . These differences in T_1 relaxation times are exploited here. As in theory, the T_1 editing approach with short TE gives better contrast-to-noise ratio, or improved lipid suppression at same acetylcarnitine signal intensities, when compared to the long TE protocol, we anticipated the T_1 editing

approach to be superior to the long TE protocol. In practice however, system stability was limiting complete suppression of the lipid signals due to subtraction artifacts with the T_1 editing approach. Therefore, the single shot, long TE protocol and subtraction-based T_1 editing protocol performed very similarly.

As an alternative strategy to use T_1 differences between acetylcarnitine and lipids, the conventional inversion recovery sequence might also be used for nulling of the lipid resonances that cover the acetylcarnitine resonance. While this approach is more sensitive to variations in T_1 relaxation times, the absence of a subtraction scheme and a higher signal intensity (80% of M_0 with IR with $TI=200$ ms vs. 65% of M_0 with the proposed T_1 editing approach) are advantageous. Initial experiments using this approach however resulted in spectra that could not be accurately phased in the acetylcarnitine region. This can be explained by the fact that in 2.2–2.4 ppm region two separate lipid resonances are contaminating the acetylcarnitine signal and these lipid resonances are characterized by different T_1 relaxation times [7]. Nulling of the 2.2 ppm resonance on one hand will lead to a negative signal component from the 2.4 ppm resonance. In contrast, with the proposed T_1 editing approach the degree of suppression increases with decreasing T_1 relaxation times for a given TI . Nulling of the 2.4 ppm resonance automatically also suppresses the shorter T_1 resonance at 2.2 ppm.

For the lean subject, with low myocellular lipid signals, lipid suppression and accurate acetylcarnitine quantification could already be achieved with the T_1 editing approach with a TI of 900 ms and with short TE of 40 ms. Importantly, the T_1 editing approach can also be combined with the long TE protocol to maximize the suppression of lipid resonances. As both low acetylcarnitine concentrations and high lipid contamination are characteristic for obese subjects and patients with type 2 diabetes (T2DM), the use of the T_1 editing approach in combination with the long TE protocol leads to enhanced suppression of the lipid signals covering the (small) acetylcarnitine peak in these subjects, enabling more accurate quantification of this peak. The power of this approach is also illustrated by a separate experiment in one of the obese subjects where we intentionally placed the voxel partially in the subcutaneous adipose tissue layer to maximize lipid contamination. The T_1 editing approach with long TE (350 ms) still resulted in complete suppression of the adipose tissue lipid signals and resulted in a well resolved acetylcarnitine peak. This peak was completely covered by lipid resonances in the long TE protocol alone.

Of course not only the lipid and acetylcarnitine signal intensities are affected by the proposed T_1 editing approach. As can be deduced from the spectra acquired with this approach, the t-Cr peak is also well preserved in the spectra. This peak can be used as an internal reference for absolute quantification of acetylcarnitine. In our experiments we estimated the signal losses on approximately 50% for t-Cr and 40% for acetylcarnitine, which is well in line with the theoretically estimated signal loss (based on a T_1 of 1100 [6] ms for t-Cr and 2000 ms for acetylcarnitine, see also figure 2). Thus, for absolute quantification, additional corrections should be applied to compensate for the T_1 induced signal losses on all the metabolites of interest. Accurate determination of the T_1 relaxation times of t-Cr and acetylcarnitine is warranted in the target patient group. However, as acetylcarnitine concentrations are changing rapidly, care has to be taken to account for these fluctuations in concentrating during the determination of relaxation times. Also, changes in T_1 and T_2 relaxation times with for example disease or after exercise, might induce quantifications errors or bias when comparing different groups. While exact data on the T_1 relaxation time of acetylcarnitine are lacking, it can be estimated that when the proposed T_1 editing approach is used, a T_1 difference of -350 ms (e.g. $T_1=1650$ ms instead of 2000 ms) would lead to an underestimation of 10% of the acetylcarnitine concentration. A T_1 difference of +500 ms (e.g. $T_1=2500$ ms instead of 2000 ms) would lead to an overestimation of around 10%. Thus, although the approach leads to heavy T_1 weighing, the intrinsic long T_1 of acetylcarnitine reduces the risk for quantification errors with changing T_1 rates. For T_2 , either a positive or negative shift of 20 ms would lead to under- or overestimation of 10%. Importantly, we have previously shown that the T_2 relaxation time for acetylcarnitine is similar for lean and obese subjects [5]. Furthermore, T_2 of acetylcarnitine did not differ pre- and post-exercise in lean subjects (data not published).

Overall, we conclude that the T_1 editing approach enhances the visibility of overlapping metabolites with a long T_1 . Using T_1 editing in addition to the long TE protocol is a feasible strategy to improve detection of the acetylcarnitine peak in subjects with high lipid signals in skeletal muscle, although signal intensity is reduced by approximately 40% when compared to the long TE protocol alone. The combination of T_1 editing with long TE optimally suppresses lipid signals and achieves near-complete cancellation of lipid signals, even in obese subjects where myocellular lipids are abundant. Importantly, as acetylcarnitine metabolism seems to be disturbed in obesity and T2DM, these patients are the most interesting population to study in that respect. The proposed technique

will hence be essential to advance the study on the significance of carnitine metabolism in obesity and T2DM. The application of the T_1 editing approach in other tissues, and in identifying metabolites that have been uncovered to date, requires future evaluation.

REFERENCES

1. Muoio DM, Noland RC, Kovalik JP, Seiler SE, Davies MN, DeBalsi KL, Ilkayeva OR, Stevens RD, Kheterpal I, Zhang J et al: Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. *Cell metabolism* 2012, 15(5):764-777.
2. Noland RC, Koves TR, Seiler SE, Lum H, Lust RM, Ilkayeva O, Stevens RD, Hegardt FG, Muoio DM: Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control. *The Journal of biological chemistry* 2009, 284(34):22840-22852.
3. Muoio DM: Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. *Cell* 2014, 159(6):1253-1262.
4. Kreis R, Jung B, Rotman S, Slotboom J, Boesch C: Non-invasive observation of acetyl-group buffering by ^1H -MR spectroscopy in exercising human muscle. *NMR in biomedicine* 1999, 12(7):471-476.
5. Lindeboom L, Nabuurs CI, Hoeks J, Brouwers B, Phielix E, Kooi ME, Hesselink MK, Wildberger JE, Stevens RD, Koves T et al: Long-echo time MR spectroscopy for skeletal muscle acetylcarnitine detection. *The Journal of clinical investigation* 2014, 124(11):4915-4925.
6. Krssak M, Mlynarik V, Meyerspeer M, Moser E, Roden M: ^1H NMR relaxation times of skeletal muscle metabolites at 3 T. *Magma* 2004, 16(4):155-159.
7. Wang L, Salibi N, Wu Y, Schweitzer ME, Regatte RR: Relaxation times of skeletal muscle metabolites at 7T. *Journal of magnetic resonance imaging : JMRI* 2009, 29(6):1457-1464.
8. Bottomley PA: Spatial localization in NMR spectroscopy in vivo. *Annals of the New York Academy of Sciences* 1987, 508:333-348.
9. Gruetter R: Automatic, localized in vivo adjustment of all first- and second-order shim coils. *Magnetic resonance in medicine* 1993, 29(6):804-811.

Chapter 6

Resveratrol improves mitochondrial function but does not affect insulin sensitivity or brown adipose tissue activity in first degree relatives of patients with type 2 diabetes

Marlies de Ligt

Yvonne M.H. Bruls

Jan Hansen

Marie-Fleur Habets

Bas Havekes

Emmani B. M. Nascimento

Esther Moonen-Kornips

Gert Schaart

Vera B. Schrauwen-Hinderling

Wouter van Marken-Lichtenbelt

Patrick A. J. Schrauwen

Published in Molecular metabolism 2018; S2212-8778(18):30221-7.

ABSTRACT

Purpose: Resveratrol improves metabolic health in healthy obese men, but not when given to patients with type 2 diabetes (T2D) as add-on therapy to metformin. Therefore, in this study we examined whether 30 days of resveratrol supplementation can enhance metabolic health in men *at risk* of developing T2D. Additionally, we examined cold-stimulated brown adipose tissue (BAT) activity, as preclinical data suggest that resveratrol could activate BAT. In a randomized placebo controlled cross-over design, thirteen male first degree relatives (FDR) of patients with T2D received resveratrol (150 mg/day) or placebo for 30 days. A sub-set of eight participants underwent an ^{18}F -FDG PET/CT scan after cold stimulation.

Results: After 30 days of resveratrol supplementation we found a significant improvement in *ex vivo* skeletal muscle mitochondrial function (state 3 respiration upon octanoyl-carnitine) measured by high-resolution respirometry. However, resveratrol supplementation did not improve insulin sensitivity, expressed as the rate of glucose disposal during a two-step hyperinsulinemic-euglycemic clamp. Also intrahepatic and intramyocellular lipid content, metabolic flexibility, substrate utilization, energy expenditure and skeletal muscle acylcarnitine profiles remained unaffected by resveratrol. ^{18}F -FDG glucose up-take in BAT remained unaffected by resveratrol. In vitro experiments in adipocytes derived from human BAT confirmed the lack of effect of resveratrol on BAT activity.

Conclusion: In conclusion, resveratrol can stimulate muscle mitochondrial function in FDR males, which is in concordance with previous results. However, no other metabolic benefits of resveratrol were found in this group of FDR males. This discrepancy could be attributed to differences in subject characteristics causing differences in metabolism of resveratrol and thereby affecting resveratrols effectiveness.

INTRODUCTION

Resveratrol, a natural polyphenol present in various foods and drinks, has been proposed as a promising treatment in the prevention and treatment of type 2 diabetes [1]. Beneficial effects of resveratrol are strongly related to the activation of sirtuin 1 (SIRT1) and AMPK-activated protein kinase (AMPK) [2]. Both proteins are important regulators of among others inflammation, cellular fuel metabolism, and mitochondrial function, thereby making them interesting targets for combating T2D [3]. Previously, we have shown that in healthy obese men 150 mg resveratrol per day for 30 days exerted beneficial health effects among which improvements in *ex vivo* mitochondrial function, energy metabolism, hepatic lipid content, and blood glucose levels [4]. However, we did not find comparable results when the same dose of resveratrol was administered to patients with type 2 diabetes mellitus (T2D) [5]. Although we confirmed beneficial effects of resveratrol on mitochondrial function, no improvement in insulin sensitivity or related health parameters was achieved. However, since these patients with T2D were using the oral-glucose lowering drug metformin, which may partly have similar targets as resveratrol, we suggested that the use of metformin might have interfered with the effectiveness of resveratrol on glucose homeostasis. To investigate if resveratrol could still have a role in the prevention of diabetes, there is a need for further studies in drug-naïve humans with compromised metabolic health. First-degree relatives of T2D patients (FDRs) display decreased beta cell and mitochondrial function and have reduced insulin sensitivity and metabolic flexibility [6-10]. Thereby, they are at increased risk of developing T2D and may specifically benefit from interventions that improve insulin sensitivity and metabolic flexibility.

Beneficial effects of resveratrol on metabolic health could also reside in the activation of brown adipose tissue (BAT) thermogenesis, as represented by increased expression of UCP1, SIRT1 and PGC-1 α in BAT of animals receiving resveratrol [11-13]. The main role of BAT is generation of heat, mediated via uncoupling protein 1 (UCP1) [14]. Activation of brown adipose tissue has been associated with positive metabolic health effects [15], but so far the effect of resveratrol on BAT activity in humans is unknown.

Therefore, in this placebo-controlled crossover study we investigated if 30 days of resveratrol supplementation can improve insulin sensitivity, metabolic flexibility and mitochondrial function in FDR males, which are at increased risk of developing T2D.

Carnitine metabolism has recently been suggested to be involved in the regulation of metabolic flexibility, and acetylcarnitine levels were found to be reduced in skeletal muscle of insulin resistant subjects. We have previously shown that resveratrol is able to affect lipid metabolism and lipid storage in skeletal muscle [4]. Therefore, we here also investigated if resveratrol could also affect acetylcarnitine levels in skeletal muscle and if these affects could underlie potential beneficial effects of resveratrol on metabolic flexibility.

Finally, in a sub-set of the participants we examined if 34 days of resveratrol supplementation is able to enhance BAT metabolism.

RESEARCH DESIGN AND METHODS

Participants

Thirteen overweight male volunteers at increased risk of developing T2D participated in the study (Supplementary Table 1). The study protocol was reviewed and approved by the institutional medical ethics committee (NL 47018.068.13). An increased risk for T2D was defined as having at least one first-degree relative with T2D, a BMI between 27-35 kg/m², and disrupted glucose homeostasis determined by a 2-hour oral glucose tolerance test (OGTT). From the OGTT, glucose clearance was calculated using the oral glucose insulin sensitivity model (OGIS₁₂₀ [16]). Participants with a glucose clearance ≤ 350 ml/kg/min were regarded as having disrupted glucose homeostasis. A sub-set of eight participants was enrolled for BAT activity measurements.

Subjects participated in a double-blind, cross-over experimental trial with two conditions: a placebo and a resVida condition (150 mg/day *trans*-resveratrol [99.9%; provided by DSM Nutritional Products Ltd.]), with a washout period of at least 30 days. Each experimental condition lasted 30 or 34 days; the 34-day protocol applied only to the sub-set of eight participants who underwent BAT measurements. Participants were instructed to take the first supplement on the day baseline measurements were performed (day 0), and the last supplement in the evening before the last test day (day 29 or day 33; depending on participation in the BAT measurement). At each visit the participants returned unused capsules, which were counted for compliance. Participants were instructed to abstain from food and beverages containing substantial amounts of resveratrol (e.g. red wine, grapes, and peanuts). Compliance with these instructions was confirmed verbally. Subjects were advised to maintain their normal eating, activity, and sleeping pattern. Subjects came on a weekly basis (days 0, 7, 14, 21, 29, and 34) to the University. The weekly checkup took place in the morning in the overnight fasted state and included a measurement of body mass and drawing of a blood sample for the analysis of resveratrol to confirm compliance to the protocol. The latter was analyzed by mass spectroscopy, as previously described [4].

General Health Parameters

On day 0 and day 30 of both the resveratrol and placebo period, fasting blood samples were drawn for analysis of general safety parameters (creatinine, bilirubin, aspartate aminotransferase, alanine aminotransferase, and γ -glutamyl transferase). In addition,

heart rate and blood pressure were measured in triplicate (Omron Healthcare, Hamburg, Germany) in the resting condition, body weight was measured and a 12-lead ECG was made. Maximal aerobic capacity ($\text{VO}_{2\text{max}}$) was measured on a cycle ergometer on day 27, to verify cardiorespiratory fitness (Omnical, Maastricht Instruments, Maastricht University). Body fat percentage was determined only once by dual energy X-ray absorptiometry (DXA, Hologic, the Netherlands) on day 0 of the first intervention period.

Cardiac Function

On day 29 in the afternoon, subjects came to the University for myocardial function measurements by Doppler ultrasound. M-mode, two-dimensional, and Doppler echocardiography were performed, using a Vivid 7 ultrasound system (GE Healthcare, Milwaukee, WI, USA) with 3.5 MHz cardiac transducer.

Magnetic Resonance Spectroscopy

On day 29, the ultrasound was followed by proton magnetic resonance spectroscopy (^1H -MRS) measurements of the liver to quantify intrahepatic lipid (IHL) content and a post-exercise phosphocreatine (PCr) recovery rate measurement, by ^{31}P -MRS, to estimate *in vivo* mitochondrial function in vastus lateralis muscle. MRS scans were performed on a 3T whole body scanner (Achieva Tx; Philips Healthcare, Best, The Netherlands). In short, for IHL a PRESS sequence was used with repetition time of 4 s and an echo time of 32.5 ms. Values are given as T2 corrected ratios of the CH_2 peak relative to unsuppressed water resonance, expressed as percentage (for further details see [17]). For *in vivo* mitochondrial function, the PCr recovery half-time, in seconds, was determined after submaximal knee-extension exercise performed in the scanner (for details see [18]). To standardize food intake, subjects had lunch with the same food items in the two conditions, and after lunch stayed fasted until the start of the measurements at 4 PM. After the MRS measurements on day 29, a standardized evening meal was provided and subjects stayed in a respiration chamber during 10 hours to allow measurement of sleeping metabolic rate [19].

Muscle Biopsy

In the morning of day 30 a biopsy was taken from the vastus lateralis muscle. A portion of the muscle was directly frozen in melting isopentane for determination of protein expression of oxidative phosphorylation (OXPHOS) by Western blotting (AB110411,

abcam, Cambridge, UK) and intramyocellular lipid content (IMCL) by Oil red O staining combined with fiber typing via immunolabeling of myosin heavy chain type I, as described [20]. Skeletal muscle acylcarnitines were analyzed as previously described using tandem mass spectrometry[21]. Total short-chain acylcarnitine species were calculated as the sum of C3 to C5 carnitine species. C6 to C12 acylcarnitine species were summed to represent medium-chain acylcarnitines and long-chain acylcarnitine species were calculated as the sum of C14 to C18-carnitine species. Another portion of 50 mg was immediately placed in ice-cold preservation medium for determination of *ex vivo* mitochondrial respiration by oxygraph (OROBOROS Instruments, Innsbruck, Austria), as previously described [4].

Hyperinsulinemic-euglycemic Clamp

On day 30, after the muscle biopsy, a two-step hyperinsulinemic-euglycemic clamp was performed to assess whole-body, and liver- and muscle-specific insulin sensitivity. The clamp started with a primed continuous infusion of D-[6,6-²H₂]glucose (0.04 mg/kg/min) to determine rates of endogenous glucose production (EGP), glucose appearance (Ra), and glucose disposal (Rd), as described earlier [5]. After 120 minutes, low dose insulin infusion was started (10 mU/m²/min) for 3 hours, followed by high dose insulin infusion (40 mU/m²/min) for 2.5 hours. During the last 30 minutes of each insulin infusion phase (0, 10, and 40 mU/m²/min), blood samples were collected, and substrate utilization was measured by indirect calorimetry (Omnicol, Maastricht Instruments, Maastricht University). Steeles single pool non-steady state equations were used to calculate glucose Ra and Rd [22]. The distribution volume of glucose was assumed to be 0.160 L/kg.

Brown Adipose Tissue Metabolism

After the clamp a sub-set of eight participants continued supplement intake for another four days. On day 34 these participants underwent an 18-Fluoro-Deoxy-Glucose (¹⁸F-FDG) PET-CT scan, upon acute cold exposure, to visualize active BAT. For defining BAT presence and activity participants were wrapped in a water-perfused suit. The protocol started with a thermoneutral period of 36°C for 45 minutes, followed by an individualized 2-hour cooling protocol. During the cooling protocol water temperature was decreased by 4°C every 15 minutes, until shivering occurred. When shivering occurred, subjects were warmed-up again for 5 minutes to diminish shivering. Finally, the suit temperature was set for a minimum of 30 minutes at 2°C above the

temperature at which shivering started. During this mild cold stimulation 74 MBq of ^{18}F -FDG was intravenously injected. One hour after injection imaging started with a low-dose CT scan (120 kV, 30 mAs), immediately followed by a static PET scan (6 to 7 bed positions, 6 min per bed position). The PET image was used to determine the ^{18}F -FDG uptake, and the CT image was used for PET attenuation correction and localization of the ^{18}F -FDG uptake sites. The scans were analyzed using PMOD software (version 3.0; PMOD Technologies). PET-active areas, defined as a standard uptake value ≥ 1.5 , were selected in the supraclavicular adipose tissue region (-200 and -10 Hounsfield units) to establish the presence and volume of active BAT. The fixed-volume method [23] was used to determine the average standard uptake value (SUV mean) and maximum standard uptake value (SUV max) of several tissues. Fixed volumes of 2.67 cm³ were placed and analyzed in supraclavicular BAT region, subcutaneous WAT, skeletal muscle, liver, and brain. The same anatomic locations were used in the analysis between the two experiments, as described earlier [23].

To examine *in vitro* effects of resveratrol on BAT oxidative capacity, BAT, and subcutaneous white adipose tissue (WAT) biopsies were obtained from patients undergoing thyroid surgery. The protocol was reviewed and approved by the institutional ethics committee (NL31367.068.10). From the collected biopsies, the stromal vascular fraction was isolated and grown to confluence before differentiation was initiated as described previously with slight modifications [24]: bFGF and BMP4 were removed from the differentiation protocol. *In vitro* differentiated adipocytes were incubated for 24 hours with *trans*-resveratrol (0, 5 and 50 μM) prior to measuring oxygen consumption in the XF96 bioanalyzer from Seahorse Bioscience. Basal oxygen consumption rate was measured, as well as norepinephrine (NE)-induced mitochondrial uncoupling. For the latter, 1 μM NE was added after blocking ATPase by addition of 2 μM oligomycin.

Statistical Analysis

Results are presented as means $\pm$ SEM when normally distributed and as median and range when this was not the case. Shapiro-Wilk normality test was performed to evaluate normality distribution. Students paired t-test was used to compare placebo and resveratrol treatment in normally distributed data; otherwise Wilcoxon signed-rank test was used. Treatment comparisons of parameters measured at the beginning and at the end of the intervention periods were assessed by two-way repeated measures ANOVA. Linear regression analyses were conducted to identify correlations between variables. A

p-value <0.05 was considered statistically significant. Statistical analyses were performed using the statistical program SPSS 22.0 for Mac OS X.

RESULTS

Study Compliance

Compliance was confirmed by analysis of plasma levels of resveratrol (free + conjugated) and dihydro-resveratrol (DHR), a metabolite of resveratrol, on a weekly basis. Levels were below detection during the placebo period, while during the resveratrol period both resveratrol and DHR levels were elevated, with resveratrol levels of 412 ± 63 ng/mL and DHR levels of 541 ± 80 ng/mL on day 30 (Figure 1).

Figure 1. Total plasma resveratrol (RSV) and dihydro-resveratrol (DHR) values during the 30 days of resveratrol treatment period.

Data are presented as mean $\pm$ SEM (n=13).

Insulin Sensitivity, Metabolic Flexibility and Substrate Kinetics

Insulin sensitivity was measured by two-step hyperinsulinemic euglycemic clamp. Basal, low- and high-insulin phase R_d were not affected by resveratrol treatment (Figure 2A). Insulin-stimulated glucose uptake, as expressed by the change in glucose disposal (ΔR_d) from basal to the low or high-insulin phase was not different between resveratrol and placebo ($p_{\text{low}}=0.507$, $p_{\text{high}}=0.810$, Figure 2B and Table 1). Indirect calorimetry, performed during the different stages of the clamp, revealed no effect of resveratrol supplementation on metabolic flexibility, expressed as the change in respiratory exchange ratio (RER) upon high insulin stimulate (ΔRER high insulin-basal). Insulin-induced glucose oxidation or suppression of fat oxidation (Table 1) were unaffected by resveratrol supplementation. Also, non-oxidative glucose disposal as well as oxidative glucose disposal remained unchanged (Table 1). Insulin mediated suppression of endogenous

glucose production (% EGP suppression) was also similar after resveratrol and placebo treatment both during the low insulin ($p>0.99$) and high insulin infusion phase ($p=0.814$) (Figure 2C and Table 1).

In accordance with a lack of effect of resveratrol on substrate utilization during the clamp, resveratrol treatment did not influence sleeping metabolic rate (5.1 ± 0.09 kJ/min during placebo, versus 5.0 ± 0.08 kJ/min during resveratrol treatment, $p=0.462$) or respiratory exchange ratio measured overnight in a respiration chamber. Furthermore, plasma FFA levels after an overnight fast were comparable between treatments ($p=0.333$) and were similarly suppressed by insulin ($p=0.550$, Table 1). In line with a lack of effect of resveratrol on insulin sensitivity and substrate utilization, no changes in fasting insulin, glucose or HbA1c levels were found upon resveratrol treatment (Supplementary Table 2). Also other plasma markers of metabolic health remained unaffected (Supplementary Table 2).

Figure 2. Effect of resveratrol (RSV) on peripheral and hepatic insulin sensitivity.

After 30 days of resveratrol and placebo, peripheral and hepatic insulin sensitivity were assessed by a two-step hyperinsulinemic-euglycemic clamp ($t=0-120$ min: D-[6,6- 2 H $_2$]glucose tracer infusion; $t=120-300$ min: low-insulin infusion; $t=300-420$ min: high-insulin infusion). Insulin-stimulated glucose disposal, expressed as the Rd and EGP were calculated for the last 30 min of the basal, low- and high-insulin state. (A) Rd and (B) difference in Rd compared to basal; (C) EGP suppression (Δ EGP%) upon low- and high-insulin infusion. Data are presented as mean $\pm$ SEM ($n=13$). Rd, rate of disappearance; EGP, endogenous glucose production.

Table 1. Insulin sensitivity and substrate kinetics

	Placebo	Resveratrol	P-value
Rd ($\mu\text{mol/kg}$ body weight/min)			
Basal	8.0 (6.86 – 9.42)	7.9 (6.86 – 8.92)	0.972a
Low insulin	10.2 $\pm$ 0.44	9.4 $\pm$ 0.56	0.258
High insulin	32.0 $\pm$ 2.17	32.0 $\pm$ 2.03	0.965
Delta (Rd low insulin - Rd basal)	1.5 (0.67 – 3.37)	0.9 (0.04 - 3.02)	0.507a
Delta (Rd high insulin - Rd basal)	23.8 $\pm$ 2.04	24.1 $\pm$ 1.90	0.810
EGP ($\mu\text{mol/kg}$ body weight/min)			
Basal	7.9 (4.48 – 9.16)	8.5 (7.29 – 9.54)	0.937a
Low insulin	3.0 (2.32 – 5.18)	3.3 (2.36 – 4.41)	0.875a
High insulin	-0.3 (-1.19 – -0.08)	-0.63 (-0.92 – -0.03)	0.695a
% suppression (low insulin)	62 (40.0 – 70.7)	58 (42.0 – 72.1)	>0.99a
% suppression (high insulin)	103 (99.8 – 113.1)	107 (100.4 – 114.6)	0.814a
NOGD ($\mu\text{mol/kg}$ body weight/min)			
Basal	2.1 $\pm$ 0.92	2.0 $\pm$ 0.65	0.894
Low insulin	2.2 $\pm$ 0.78	1.0 $\pm$ 0.88	0.350
High insulin	18.1 $\pm$ 2.11	17.6 $\pm$ 5.91	0.742
Carbohydrate oxidation ($\mu\text{mol/kg}$ body weight/min)			
Basal	5.3 (4.60 – 7.47)	5.9 (4.27 – 7.38)	0.944a
Low insulin	7.9 $\pm$ 0.53	8.4 $\pm$ 0.76	0.630
High insulin	13.9 $\pm$ 0.51	14.4 $\pm$ 0.95	0.478
Delta (low insulin - basal)	1.9 $\pm$ 0.54	2.6 $\pm$ 0.61	0.343
Delta (high insulin - basal)	7.9 $\pm$ 0.81	8.6 $\pm$ 0.97	0.328
FFA oxidation ($\mu\text{mol/kg}$ body weight/min)			
Basal	3.5 $\pm$ 0.24	3.6 $\pm$ 0.13	0.978
Low insulin	2.9 $\pm$ 0.17	2.7 $\pm$ 0.14	0.527
High insulin	1.6 $\pm$ 0.13	1.5 $\pm$ 0.18	0.757
Delta (low insulin - basal)	-0.64 $\pm$ 0.15	-0.80 $\pm$ 0.15	0.355
Delta (high insulin - basal)	-1.98 $\pm$ 0.19	-2.05 $\pm$ 0.203	0.694
Plasma FFA ($\mu\text{mol/L}$)			
Basal	552 $\pm$ 40.2	609 $\pm$ 59.9	0.333
Low insulin	167 $\pm$ 12.2	174 $\pm$ 19.7	0.652
High insulin	66 $\pm$ 5.3	65 $\pm$ 6.3	0.550
Respiratory Exchange Ratio			
Basal	0.77 (0.765 – 0.811)	0.78 (0.766– 0.805)	0.916a
Low insulin	0.82 $\pm$ 0.008	0.82 $\pm$ 0.009	0.634
High insulin	0.90 $\pm$ 0.008	0.90 $\pm$ 0.011	0.833
Delta (low insulin - basal)	0.03 $\pm$ 0.008	0.04 $\pm$ 0.008	0.241
Delta (high insulin - basal)	0.11 $\pm$ 0.010	0.12 $\pm$ 0.013	0.389

Substrate metabolism assessed by a two-step hyperinsulinemic euglycemic clamp after 30 days of placebo and resveratrol supplementation. Glucose oxidation, lipid oxidation, and respiratory quotient were determined by means of indirect calorimetry. Non-oxidative glucose disposal (NOGD) is calculated by subtracting the rate of glucose oxidation from the rate of disappearance of the glucose (R_d glucose). Data are presented as mean $\pm$ SEM when normally distributed, otherwise median and range are shown ($n=13$). ^aP-value relates to non-parametric Wilcoxon Signed Rank test. R_d , rate of disappearance; EGP, endogenous glucose production; NOGD, non-oxidative glucose disposal; FFA, free fatty acid.

Mitochondrial Function

Resveratrol is expected to act on whole body metabolism via the improvement of mitochondrial function. We therefore measured mitochondrial state 3 respiration on a lipid-derived substrate (malate + octanoyl-carnitine, 3MO). State 3 respiration was significantly higher after resveratrol supplementation (32 ± 1.8 after placebo vs. 37 ± 1.9 pmol/mg protein/s after resveratrol, $p < 0.001$; Figure 3A). Also, state 3 respiration with parallel electron input to both complex I and II (malate + octanoyl-carnitine + glutamate + succinate, 3MOGS) was significantly higher after resveratrol supplementation (73 ± 3.7 after placebo vs. 78 ± 4.3 pmol/mg protein/s after resveratrol, $p = 0.050$; Figure 3A). These results confirm previous reported results for a stimulatory effect of resveratrol on skeletal muscle mitochondrial function [4, 5]. Maximal FCCP-induced uncoupled respiration (state u) seemed to be increase upon resveratrol, but failed to reach statistical significance ($p = 0.218$, Figure 3B). In the absence of the lipid-derived substrate octanoyl-carnitine, no effects of resveratrol were observed in state 3 (3MG, 3MGS) or state 4o respiration with complex I- and II-linked substrates (Figure 3C, D), consistent with our previous reports [4, 5]. Protein content of the structural components of the individual OXPHOS complexes remained unaffected, except for complex IV that was significantly lower expressed after resveratrol supplementation ($p = 0.033$, Figure 3E).

Finally, *in vivo* mitochondrial function, determined by PCr recovery half-time, was unchanged by resveratrol compared to placebo ($p = 0.386$, Figure 3F). Correspondingly, VO_{2max} was not affected by resveratrol supplementation (27 ± 1.5 mL/kg bw/min after placebo vs. 28 ± 1.7 after resveratrol, $p = 0.823$).

Figure 3. Effect of resveratrol (RSV) on *ex vivo* and *in vivo* mitochondrial function.

After 30 days of resveratrol and placebo, a muscle biopsy specimen was obtained from the vastus lateralis muscle. Part of the specimen was used for evaluation of *ex vivo* mitochondrial function (n=10). A: ADP stimulated respiration (state 3) upon a lipid-like substrate and upon parallel electron input into complex I and II. B: Maximally uncoupled respiration upon FCCP (carbonyl cyanide p-(trifluoromethoxy)-phenylhydrazine). C: ADP stimulated respiration (state 3) upon parallel electron input into complex I and II. D: mitochondrial respiration uncoupled from ATP synthesis (state 4_o). E: the protein content of the individual complexes of the electron transport chain is quantified by western blotting in vastus lateralis muscle. An antibody cocktail that detects all five complexes was used (n=10). F: *in vivo* mitochondrial function expressed as PCr half-time (n=9). Data are presented as means ± SEM. *P<0.05 compared to placebo. M, malate; O, octanoyl-carnitine; G, glutamate; S, succinate; OXPHOS, oxidative phosphorylation; PCr, phosphocreatine recovery

Brown Adipose Tissue Activity

Rodent studies indicated that resveratrol could activate BAT. Here, we examined if resveratrol treatment increases cold-activated BAT activity using PET-CT scanning on day 34, in a sub-set of eight participants. Only one participant showed BAT activity above the threshold of 1.5 SUV in both periods, as can be clearly seen in Figure 4A as the top line. Using the fixed volume method, we found that both BAT SUVmax (1.6 ± 0.82 and 1.9 ± 0.61 , $p=0.502$, Figure 4A) and BAT SUVmean (1.1 ± 0.62 and 1.0 ± 0.38 after placebo and resveratrol respectively, $p=0.734$, Figure 4B) were not changed after resveratrol. Likewise, ^{18}F -FDG uptake did not change in skeletal muscle,

subcutaneous white adipose tissue (WAT), or liver (Figure 4B). In accordance with a lack of effect of resveratrol on BAT activity, resveratrol treatment did not influence cold-induced metabolic rate (5.3 ± 0.22 kJ/min during placebo, versus 5.4 ± 0.22 kJ/min during resveratrol treatment, $p=0.817$).

In our human clinical trial, the treatment duration may have been too short or the dose of resveratrol too low to accomplish effects on BAT activity. Therefore, to further investigate if resveratrol can stimulate human BAT metabolism, we examined mitochondrial uncoupling in adipocytes derived from human BAT and WAT. NE-stimulated mitochondrial uncoupling was specifically increased in adipocytes derived from human BAT. However, 5 or 50 μ M *trans*-resveratrol did not alter NE-stimulated mitochondrial uncoupling in adipocytes derived from BAT or WAT (Figure 4C, D).

Figure 4. Effects of resveratrol (RSV) on *in vivo* and *in vitro* brown adipose tissue metabolism.

A-B: ^{18}F -FDG uptake in BAT, WAT, muscle, and liver after 34 days of placebo and resveratrol intervention ($n=8$). BAT SUV max (A) and BAT SUV mean (B) were measured under cold-stimulated conditions. (C-D) Respiration was measured in oligomycin (OG)-treated brown ($n=4$) and white adipocytes ($n=6$) following 1 μ M norepinephrine. Data are presented as mean $\pm$ SEM. BAT, brown adipose tissue; WAT, white adipose tissue; SUV, standard uptake value; OCR, oxygen consumption rate.

Ectopic Fat Accumulation

IHL content, determined by ^1H -MRS, remained unaffected by 30 days of resveratrol supplementation (Figure 5A). Also, IMCL, analyzed *ex vivo* in the muscle biopsies, revealed no change in lipid area fraction in neither type I or type II muscle fibers (Figure 5B).

Figure 5. Effect of resveratrol (RSV) on ectopic lipid storage.

A: Intrahepatic lipid content quantified by ^1H -MRS after 29 days of resveratrol and placebo supplementation. Box plot represents minimum, first quartile, median, third quartile, and maximum ($n=10$). B: Muscle biopsy sections for the vastus lateralis muscle were stained for intramyocellular lipid content by Oil Red O staining. Intramyocellular lipid content is quantified as the percentage area of a muscle fiber that is covered by lipids. Data are presented as means $\pm$ SEM ($n=9$).

Skeletal Muscle Acylcarnitine Profiles

Acylcarnitine profiles in skeletal muscle tissue were assessed in a muscle biopsy taken after an overnight fast. Resveratrol treatment did not affect free carnitine availability ($p=0.349$, figure 6A) or acetylcarnitine concentrations ($p=0.672$, figure 6B) in skeletal muscle tissue. Short-chain, medium-chain and long-chain acylcarnitines remained unaltered upon 30 days of resveratrol treatment ($p>0.05$, figure 6CDE). The concentrations of individual acylcarnitine species are reported in supplementary table 3.

Cardiac Function

Systolic and diastolic blood pressure was unaltered by resveratrol ($p=0.40$ and $p=0.76$ respectively, Supplementary Table 3). Also, no changes in stroke volume, cardiac output, left ventricular end systolic diameter, or left ventricular ejection fraction were found (Supplementary Table 3). Parameters of diastolic function and parameters representing structural changes of the heart also remained unchanged (Supplementary Table 4).

Figure 6. Effect of resveratrol (RSV) on skeletal muscle acylcarnitine profiles.

After 30 days of resveratrol and placebo, a muscle biopsy specimen was obtained from the vastus lateralis muscle. Part of the specimen was used for quantification of skeletal muscle acylcarnitine profiles by tandem mass spectrometry ($n=13$). A: free carnitine concentrations, B acetylcarnitine concentrations, C: short-chain acylcarnitines calculated as the sum of C₃ to C₅ carnitine species, D: C₆ to C₁₂ acylcarnitine species were summed to represent medium-chain acylcarnitines and E: long-chain acylcarnitine concentrations species calculated as the sum of C₁₄-C₁₈-carnitine species. Data are presented as means $\pm$ SEM.

DISCUSSION

Resveratrol has been suggested to have beneficial effects in the prevention and treatment of T2D. Here, we investigated the effects of 30 days of resveratrol supplementation in men at increased risk of developing T2D. However, in this population we did not see an improvement in insulin sensitivity after 30 days of 150 mg/day resveratrol compared to placebo. Neither hepatic nor peripheral insulin sensitivity were improved, albeit we did demonstrate that 150 mg resveratrol per day was sufficient to improve *ex vivo* muscle mitochondrial oxidative capacity. Other markers of metabolic health and BAT metabolism also remained unchanged. Our data indicates that low-dose resveratrol supplementation does not seem to have beneficial metabolic health effects in men at increased risk of developing T2D.

The potential role resveratrol could play in prevention and treatment of metabolic diseases, such as T2D, has been widely studied over the past decades. Studies in rodents show clear improvement upon resveratrol administration on among others insulin sensitivity, mitochondrial function, liver fat accumulation, and indications towards increased BAT metabolism [25]. Nevertheless, human clinical trials have given inconsistent results so far. Some studies show clear improvement in parameters related to metabolic health [4, 26-30], while others see no or barely any effects [5, 31-37].

Here, we used the same dose and treatment duration of resveratrol as in our first study in healthy obese volunteers. In that study, we found beneficial effects of 30 days of 150 mg/day resveratrol on several health parameters, but most importantly activation of the SIRT1 - PGC-1 α pathway in skeletal muscle, illustrating that resveratrol at this dose is indeed capable of affecting this pathway in humans [4]. However, three other studies, with comparable resveratrol dose and treatment duration, failed to achieve improvements in glucose control [5, 36, 37]. Though, we have consistently demonstrated that resveratrol at least affects mitochondrial metabolism in skeletal muscle [4, 5]. Denoting that resveratrol does have the expected effects on skeletal muscle. One can therefore debate whether the dose of resveratrol used might be too low or the treatment duration too short to elicit effects on glucose metabolism in patients or subjects with disrupted glucose homeostasis. Though, a recent clinical trial by Kjaer et al. [38] used a high (1000 mg/day) and low dose (150 mg/day) of resveratrol for a period of six months and also reported no effects on glucose or lipid metabolism in obese men with

moderate insulin resistance. It can therefore be speculated that differences in subject characteristics, such as age, body weight, gut microbiota composition, and metabolic status are actually the confounding factors.

It is known that resveratrol is rapidly metabolized into its glucuronidated and sulfated form in the intestine and liver, resulting in a very low bioavailability [39]. In addition, a recent study suggest that unconjugated resveratrol, also called free resveratrol, is the active form of resveratrol [40]. Therefore, measuring plasma concentrations of free and conjugated resveratrol could shed light on why resveratrol is effective in some but not in other studies. Unfortunately, few clinical trials have actually measured plasma resveratrol concentrations. In studies that do report resveratrol levels, plasma concentrations of free resveratrol are generally below detection level, even after resveratrol has been consumed as a supplement [4, 5, 36]. Therefore we compared our plasma resveratrol concentrations to two of our previous studies that used the same dose and duration of resveratrol treatment: one study with healthy obese men [4] and one study with T2D patients [5]. In all three studies, we observed positive effects of resveratrol on mitochondrial metabolism. Whereas the study with healthy obese men gave positive effects on glucose metabolism, our current study in FDRs and our previous study in T2D patients did not. Strikingly, in the study with healthy obese men, total resveratrol concentrations (free + conjugated) were significantly lower than those measured in the study with T2D patients and in our current FDRs study (healthy obese: 183 ± 30 ng/mL; T2D patients: 379 ± 41 ng/mL; FDRs: 412 ± 63 ng/mL, $p < 0.01$). These relatively high plasma levels of resveratrol in our FDR participants could be caused by several factors: increased uptake of resveratrol from the gut, increased conjugation of resveratrol, diminished excretion of resveratrol by the kidneys and/or less uptake of resveratrol from the plasma by the tissues. Over the 30-day resveratrol treatment period no increase was observed in free resveratrol or DHR, indicating no accumulation occurred in the plasma. This suggests the excretion by the kidneys was probably not hampered.

With respect to the uptake from the plasma by the tissues, Lanon et al. revealed that human hepatic cell uptake of resveratrol can be affected by serum proteins [41]. It has been shown that resveratrol can be trapped in serum constituents, particularly albumin [41, 42]. Considering there is an association between serum albumin levels and insulin resistance, the T2D patients and FDR participants could have had increased serum

albumin levels in turn affecting bioavailability of resveratrol. This hypothesis however requires further exploration. Specific attention should be paid to uptake mechanisms of resveratrol in tissues, since this is still largely unknown.

It is also possible that more resveratrol was conjugated by liver and gut. It is known that conjugation of compounds can be influenced by age, sex, and genetics [43]. In that respect, it is important to note that the participants in the T2D and FDR study were significantly older by 10-13 years than those enrolled in the healthy obese trial. These differences in age could have caused changes in conjugation rate of resveratrol and thereby decreased levels of free and thereby biological active resveratrol, but this hypothesis needs to be explored further.

In our current study, we found significant improvements in mitochondrial state 3 respiration on a lipid-derived substrate. These results are comparable to previous clinical trials [4, 5, 44]. We noted these improvements without changes in OXPHOS protein content, except for a decrease in complex IV, indicating that resveratrol affects predominantly mitochondrial efficiency and not abundance. An alternative explanation for the lack of effect of resveratrol on metabolic health parameters is that merely improving mitochondrial function is not sufficient to improve whole body insulin sensitivity or metabolic health in general. This suggestion is supported by findings of Hoehn et al. [45], who concluded that acute or chronic up-regulation of mitochondrial fatty acid oxidation has no net effect on whole-body energy expenditure or adiposity. In addition, Wong et al. [46] previously reported that overexpressing PGC-1 α in mouse skeletal muscle did not prevent high-fat diet induced insulin resistance, despite improved mitochondrial function. Only when mice were stimulated to become physical active, the PGC-1 α induced increase in mitochondrial function was also accompanied by improved insulin sensitivity. This may suggest that just increasing mitochondrial function is not sufficient to stimulate insulin sensitivity and it could be speculated that physical activity levels in our T2D patients [4] or FDR subjects were too low to benefit from the small increase in resveratrol-induced mitochondrial function. However, this concept would have to be tested in future human intervention trials.

Skeletal muscle free carnitine concentrations have previously been reported to be reduced in metabolic inflexibility and insulin resistance rodents, whereas long-chain acylcarnitine have been reported to be elevated [47-52]. Combined resveratrol and

epigallocatechin-3-gallate treatment was shown to improve metabolic flexibility and increase lipid oxidation upon a high fat mixed meal challenge [44]. Whether positive change in skeletal muscle acylcarnitine species might have contributed to these beneficial effects on metabolic flexibility remains elusive. Therefore, we here hypothesized that resveratrol treatment could beneficially affect acylcarnitine species in skeletal muscle tissue, thereby contributing to metabolic flexibility and insulin sensitivity. However, we did not detect differences in skeletal muscle acylcarnitines upon resveratrol treatment in FDR subjects, and in line with the latter, resveratrol treatment did not affect metabolic flexibility or insulin sensitivity. Therefore, whether acetylarnitine metabolism is involved in the beneficial effects of resveratrol that has been reported in some studies, could unfortunately not be revealed.

Brown adipocytes contain a high density of mitochondria and have been suggested to also be a target of resveratrol. Pre-clinical studies have indeed shown that resveratrol can increase expression of UCP1, SIRT1, and PGC-1 α in BAT and thereby increase thermogenesis [11-13]. This in turn could have beneficial effects on energy expenditure and could ultimately result in weight loss [11-13]. This principle has, to our knowledge, never been examined *in vivo* in humans. Unfortunately, we were unable to detect differences in BAT activity measured by cold-stimulated ^{18}F -FDG uptake after 34 days of supplementation with resveratrol. In this regard, the age of the participants could have played a role since BAT activity declines with age and is decreased in males and overweight [53]. The supplementation period could have been too short to recruit new BAT. Correspondingly, when *in vitro* cultured adipocytes derived from human BAT were incubated with 5 or 50 μM *trans*-resveratrol this did not increase NE-stimulated mitochondrial uncoupling.

In conclusion, the current study indicates that resveratrol does not improve insulin sensitivity, metabolic flexibility, substrate utilization, energy expenditure, ectopic fat accumulation, skeletal muscle acylcarnitine profiles, or cardiac function, and does not stimulate BAT metabolism in men at increased risk of developing T2D. However, the current study does support our previous findings that low-dose resveratrol supplementation can improve muscle mitochondrial oxidative capacity. Future studies should unravel why there are inconsistencies in effectiveness of resveratrol when supplied to different populations, which may include looking into physical activity level of participants and bioavailability and active compounds of resveratrol.

REFERENCES

1. Szkudelski, T. and K. Szkudelska, Resveratrol and diabetes: from animal to human studies. *Biochim Biophys Acta*, 2015. 1852(6): p. 1145-54.
2. Kulkarni, S.S. and C. Canto, The molecular targets of resveratrol. *Biochim Biophys Acta*, 2015. 1852(6): p. 1114-23.
3. Ruderman, N.B., et al., AMPK and SIRT1: a long-standing partnership? *Am J Physiol Endocrinol Metab*, 2010. 298(4): p. E751-60.
4. Timmers, S., et al., Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans. *Cell Metab*, 2011. 14(5): p. 612-22.
5. Timmers, S., et al., Resveratrol as Add-on Therapy in Subjects With Well-Controlled Type 2 Diabetes: A Randomized Controlled Trial. *Diabetes Care*, 2016. 39(12): p. 2211-2217.
6. Vaag, A., et al., Metabolic impact of a family history of Type 2 diabetes. Results from a European multicentre study (EGIR). *Diabet Med*, 2001. 18(7): p. 533-40.
7. Patti, M.E., et al., Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proceedings of the National Academy of Sciences of the United States of America*, 2003. 100(14): p. 8466-8471.
8. Morino, K., et al., Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J Clin Invest*, 2005. 115(12): p. 3587-93.
9. Fernandez-Castaner, M., et al., Beta-cell dysfunction in first-degree relatives of patients with non-insulin-dependent diabetes mellitus. *Diabet Med*, 1996. 13(11): p. 953-9.
10. Phielix, E., et al., Lower intrinsic ADP-stimulated mitochondrial respiration underlies in vivo mitochondrial dysfunction in muscle of male type 2 diabetic patients. *Diabetes*, 2008. 57(11): p. 2943-9.
11. Lagouge, M., et al., Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1 α . *Cell*, 2006. 127(6): p. 1109-22.
12. Alberdi, G., et al., Thermogenesis is involved in the body-fat lowering effects of resveratrol in rats. *Food Chem*, 2013. 141(2): p. 1530-5.
13. Andrade, J.M., et al., Resveratrol increases brown adipose tissue thermogenesis markers by increasing SIRT1 and energy expenditure and decreasing fat accumulation in adipose tissue of mice fed a standard diet. *Eur J Nutr*, 2014.

14. Cannon, B. and J. Nedergaard, Brown adipose tissue: function and physiological significance. *Physiol Rev*, 2004. 84(1): p. 277-359.
15. Schulz, T.J. and Y.H. Tseng, Brown adipose tissue: development, metabolism and beyond. *Biochem J*, 2013. 453(2): p. 167-78.
16. Mari, A., et al., A model-based method for assessing insulin sensitivity from the oral glucose tolerance test. *Diabetes Care*, 2001. 24(3): p. 539-48.
17. Lindeboom, L., et al., Proton magnetic resonance spectroscopy reveals increased hepatic lipid content after a single high-fat meal with no additional modulation by added protein. *Am J Clin Nutr*, 2015. 101(1): p. 65-71.
18. Schrauwen-Hinderling, V.B., et al., Impaired in vivo mitochondrial function but similar intramyocellular lipid content in patients with type 2 diabetes mellitus and BMI-matched control subjects. *Diabetologia*, 2007. 50(1): p. 113-20.
19. Schoffelen, P.F., et al., A dual-respiration chamber system with automated calibration. *J Appl Physiol*, 1997. 83(6): p. 2064-72.
20. Koopman, R., G. Schaart, and M.K. Hesselink, Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem Cell Biol*, 2001. 116(1): p. 63-8.
21. van Vlies, N., et al., Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. *Biochem J*, 2005. 387(Pt 1): p. 185-93.
22. Steele, R., Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann N Y Acad Sci*, 1959. 82: p. 420-30.
23. van der Lans, A.A., et al., Cold-activated brown adipose tissue in human adults: methodological issues. *Am J Physiol Regul Integr Comp Physiol*, 2014. 307(2): p. R103-13.
24. Broeders, E.P., et al., The Bile Acid Chenodeoxycholic Acid Increases Human Brown Adipose Tissue Activity. *Cell Metab*, 2015. 22(3): p. 418-26.
25. de Ligt, M., S. Timmers, and P. Schrauwen, Resveratrol and obesity: Can resveratrol relieve metabolic disturbances? *Biochim Biophys Acta*, 2015. 1852(6): p. 1137-44.
26. Bhatt, J.K., S. Thomas, and M.J. Nanjan, Resveratrol supplementation improves glycemic control in type 2 diabetes mellitus. *Nutr Res*, 2012. 32(7): p. 537-41.
27. Brasnyo, P., et al., Resveratrol improves insulin sensitivity, reduces oxidative stress and activates the Akt pathway in type 2 diabetic patients. *Br J Nutr*, 2011. 106(3): p. 383-9.

28. Crandall, J.P., et al., Pilot study of resveratrol in older adults with impaired glucose tolerance. *J Gerontol A Biol Sci Med Sci*, 2012. 67(12): p. 1307-12.
29. Movahed, A., et al., Antihyperglycemic effects of short term resveratrol supplementation in type 2 diabetic patients. *Evid Based Complement Alternat Med*, 2013. 2013: p. 851267.
30. Mendez-del Villar, M., et al., Effect of resveratrol administration on metabolic syndrome, insulin sensitivity, and insulin secretion. *Metab Syndr Relat Disord*, 2014. 12(10): p. 497-501.
31. Yoshino, J., et al., Resveratrol supplementation does not improve metabolic function in nonobese women with normal glucose tolerance. *Cell Metab*, 2012. 16(5): p. 658-64.
32. Dash, S., et al., High-dose resveratrol treatment for 2 weeks inhibits intestinal and hepatic lipoprotein production in overweight/obese men. *Arterioscler Thromb Vasc Biol*, 2013. 33(12): p. 2895-901.
33. Poulsen, M.M., et al., High-dose resveratrol supplementation in obese men: an investigator-initiated, randomized, placebo-controlled clinical trial of substrate metabolism, insulin sensitivity, and body composition. *Diabetes*, 2013. 62(4): p. 1186-95.
34. Chachay, V.S., et al., Resveratrol Does Not Benefit Patients With Nonalcoholic Fatty Liver Disease. *Clin Gastroenterol Hepatol*, 2014.
35. Olesen, J., et al., Exercise training, but not resveratrol, improves metabolic and inflammatory status in skeletal muscle of aged men. *J Physiol*, 2014. 592(8): p. 1873-86.
36. van der Made, S.M., J. Plat, and R.P. Mensink, Resveratrol does not influence metabolic risk markers related to cardiovascular health in overweight and slightly obese subjects: a randomized, placebo-controlled crossover trial. *PLoS One*, 2015. 10(3): p. e0118393.
37. Zortea, K., et al., Resveratrol Supplementation in Schizophrenia Patients: A Randomized Clinical Trial Evaluating Serum Glucose and Cardiovascular Risk Factors. *Nutrients*, 2016. 8(2): p. 73.
38. Kjaer, T.N., et al., No Beneficial Effects of Resveratrol on the Metabolic Syndrome: A Randomized Placebo-Controlled Clinical Trial. *J Clin Endocrinol Metab*, 2017. 102(5): p. 1642-1651.
39. Walle, T., Bioavailability of resveratrol. *Ann N Y Acad Sci*, 2011. 1215: p. 9-15.
40. Gheldof, N., et al., Role of sulfotransferases in resveratrol metabolism in human adipocytes. *Mol Nutr Food Res*, 2017.

41. Lancon, A., et al., Human hepatic cell uptake of resveratrol: involvement of both passive diffusion and carrier-mediated process. *Biochem Biophys Res Commun*, 2004. 316(4): p. 1132-7.
42. Jannin, B., et al., Transport of resveratrol, a cancer chemopreventive agent, to cellular targets: plasmatic protein binding and cell uptake. *Biochem Pharmacol*, 2004. 68(6): p. 1113-8.
43. Liston, H.L., J.S. Markowitz, and C.L. DeVane, Drug glucuronidation in clinical psychopharmacology. *J Clin Psychopharmacol*, 2001. 21(5): p. 500-15.
44. Most, J., et al., Combined epigallocatechin-3-gallate and resveratrol supplementation for 12 wk increases mitochondrial capacity and fat oxidation, but not insulin sensitivity, in obese humans: a randomized controlled trial. *Am J Clin Nutr*, 2016. 104(1): p. 215-27.
45. Hoehn, K.L., et al., Acute or chronic upregulation of mitochondrial fatty acid oxidation has no net effect on whole-body energy expenditure or adiposity. *Cell Metab*, 2010. 11(1): p. 70-6.
46. Wong, K.E., et al., Muscle-Specific Overexpression of PGC-1alpha Does Not Augment Metabolic Improvements in Response to Exercise and Caloric Restriction. *Diabetes*, 2015. 64(5): p. 1532-43.
47. Koves, T.R., et al., Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab*, 2008. 7(1): p. 45-56.
48. Liepinsh, E., et al., Decreased acylcarnitine content improves insulin sensitivity in experimental mice models of insulin resistance. *Pharmacol Res*, 2016. 113(Pt B): p. 788-795.
49. Muoio, D.M., Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. *Cell*, 2014. 159(6): p. 1253-62.
50. Muoio, D.M., et al., Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. *Cell Metab*, 2012. 15(5): p. 764-77.
51. Noland, R.C., et al., Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control. *J Biol Chem*, 2009. 284(34): p. 22840-52.
52. Stephens, F.B., D. Constantin-Teodosiu, and P.L. Greenhaff, New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *J Physiol*, 2007. 581(Pt 2): p. 431-44.
53. Lecoultre, V. and E. Ravussin, Brown adipose tissue and aging. *Curr Opin Clin Nutr Metab Care*, 2011. 14(1): p. 1-6.

SUPPLEMENTARY DATA

Supplementary Table. 1. Baseline characteristics

Parameter	
Age, years	66 (59.2 – 67.6)
Height, m	1.77 ± 0.012
Body weight, kg	90.9 ± 1.43
BMI, kg/m ²	28.9 ± 0.38
Body fat, %	29.0 ± 0.87
Glucose clearance, ml/kg/min	334 (303.0 – 340.3)
First-degree relatives diabetes with T2D, n	1 ± 0.2

Subjects characteristics at screening. Values are given as means ± SEM for normal distributed data, and as median (95% CI) when this is not the case (n=13)

Supplementary Table. 2. Plasma biochemistry before and after 30 days of resveratrol and placebo

	Placebo	Resveratrol	P-value
Glucose , mmol/l			
baseline	5.7 ± 0.14 ^a	5.9 ± 0.18	0.408
30 days	5.5 ± 0.17	5.6 ± 0.17	
HbA1_c , %			
baseline	5.7 ± 0.09 ^a	5.6 ± 0.10	0.326
30 days	5.7 ± 0.09	5.6 ± 0.07	
HbA1_c , mmol/mol			
baseline	39 ± 0.9	37 ± 1.1	0.354
30 days	38 ± 1.0	38 ± 0.8	
Insulin , pmol/l			
baseline	71.8 ± 8.24	78.2 ± 5.85	0.948
30 days	76.3 ± 9.33	83.3 ± 10.15	
Potassium			
baseline	4.46 ± 0.05	4.45 ± 0.05	0.976
30 days	4.25 ± 0.06	4.23 ± 0.05	
Urea			
baseline	5.3 ± 0.28	5.5 ± 0.30	0.293
30 days	4.7 ± 0.15	4.5 ± 0.18	
Creatinine , μmol/l			
baseline	89 ± 2.3	87 ± 1.8 ^a	0.764
30 days	84 ± 1.8	84 ± 2.1	
GGT , U/l			
baseline	26 ± 2.7	23 ± 1.9	0.033
30 days	23 ± 3.1 ^a	22 ± 2.2 ^a	
AST , U/l			
baseline	24 ± 1.9	23 ± 1.7 ^a	0.208
30 days	22 ± 2.1	22 ± 1.6	

ALT, U/l			
baseline	22 (19.6 – 34.4) ^b	21 (18.3 – 27.8) ^b	0.173 ^b
30 days	17 (15.7 – 30.5) ^b	23 (16.4 – 30.3) ^b	
Bilirubin, $\mu\text{mol/l}$			
baseline	9.1 $\pm$ 0.97	9.2 $\pm$ 1.11	0.959
30 days	10.5 $\pm$ 0.97	10.6 $\pm$ 0.79	
Cholesterol, mmol/l			
baseline	5.8 $\pm$ 0.29	5.5 $\pm$ 0.28	0.012
30 days	5.3 $\pm$ 0.23	5.5 $\pm$ 0.27	
HDL-cholesterol, mmol/l			
baseline	1.3 $\pm$ 0.09	1.3 $\pm$ 0.09	0.614
30 days	1.2 $\pm$ 0.09	1.2 $\pm$ 0.08	
LDL-cholesterol, mmol/l			
baseline	3.9 $\pm$ 0.22	3.6 $\pm$ 0.24	0.120
30 days	3.4 $\pm$ 0.16	3.5 $\pm$ 0.27	
Triglycerides, mmol/l			
baseline	1.40 (1.20 – 1.77)	0.98 (0.93 – 1.70) ^b	0.30 ^b
30 days	1.29 (1.12 – 1.81)	1.32 (1.19 – 2.04)	
Free fatty acids, mmol/l			
baseline	0.50 $\pm$ 0.07	0.52 $\pm$ 0.08	0.731
30 days	0.70 $\pm$ 0.06	0.75 $\pm$ 0.09	

Data are presented as mean $\pm$ SEM when normally distributed, otherwise median and range are shown (n=13). P-value reflects time*treatment effect by two-way repeated measures ANOVA. Plasma values are obtained after an overnight fast. ^aData has a non-normal distribution, but residuals from the ANOVA fit are normally distributed. ^bData and residuals are not normal distributed, analyses performed on log transformed data. GGT, γ -glutamyl transferase; AST, aspartate transaminase; ALT, alanine transaminase.

Supplementary Table. 3. Skeletal muscle acylcarnitine profiles before and after 30 days of resveratrol and placebo treatment

Skeletal muscle acylcarnitines (pmol/mg dry weight)	Placebo	Resveratrol	P-value
C0	4807.83 $\pm$ 454.80	4319.72 $\pm$ 415.32	0.349
C2	999.76 $\pm$ 197.17	889.71 $\pm$ 127.93	0.672
C3	17.69 $\pm$ 1.36	18.39 $\pm$ 1.67	0.947
C4	13.12 $\pm$ 1.67	12.19 $\pm$ 1.87	0.757
C5:1	0.78 $\pm$ 0.13	0.61 $\pm$ 0.11	0.297
C5	4.64 $\pm$ 0.53	4.47 $\pm$ 0.56	0.759
C4-3OH	4.11 $\pm$ 1.02	3.49 $\pm$ 0.70	0.361
C6	4.87 $\pm$ 0.84	3.97 $\pm$ 0.68	0.431
C8:1	1.01 $\pm$ 0.14	0.95 $\pm$ 0.09	0.687
C8	2.60 $\pm$ 0.44	2.07 $\pm$ 0.29	0.380
C4DC	1.40 $\pm$ 0.09	1.22 $\pm$ 0.09	0.157
C10	1.66 $\pm$ 0.30	1.36 $\pm$ 0.23	0.513
C12:1	0.70 $\pm$ 0.15	0.52 $\pm$ 0.07	0.266
C12	2.69 $\pm$ 0.64	1.88 $\pm$ 0.37	0.294
C14:2	1.88 $\pm$ 0.46	1.29 $\pm$ 0.29	0.246
C14:1	6.88 $\pm$ 1.84	4.47 $\pm$ 1.00	0.258
C14	6.93 $\pm$ 1.83	5.41 $\pm$ 1.57	0.465
C16:2	1.84 $\pm$ 0.43	1.51 $\pm$ 0.32	0.393
C16:1	7.16 $\pm$ 1.91	5.36 $\pm$ 1.33	0.397
C16	13.53 $\pm$ 3.18	14.83 $\pm$ 3.04	0.860
C18:2	4.20 $\pm$ 0.78	4.78 $\pm$ 0.83	0.711
C18:1	14.34 $\pm$ 3.04	14.97 $\pm$ 2.55	0.988
C18	4.36 $\pm$ 0.88	4.66 $\pm$ 0.84	0.962
C20:2	0.10 $\pm$ 0.02	0.09 $\pm$ 0.02	0.425
C20:1	0.21 $\pm$ 0.03	0.19 $\pm$ 0.03	0.620
C20	0.09 $\pm$ 0.02	0.07 $\pm$ 0.01	0.547

Skeletal muscle acylcarnitine concentrations after 30 days of placebo and resveratrol supplementation. Data are presented as mean $\pm$ SEM (n=13).

Supplementary Table. 4. Cardiac function with echocardiography

	Placebo	Resveratrol	P-value
Blood pressure and heart rate			
HR, bpm	61 ± 1.9	62 ± 2.2	0.653
Systolic blood pressure, mmHg	136 ± 3.0	138 ± 2.2	0.391
Diastolic blood pressure, mmHg	86 ± 1.8	87 ± 2.1	0.776
LV systolic function			
SV, ml	86 ± 7.8	84 ± 6.7	0.832
CO, L/min	6.0 ± 0.64	5.8 ± 0.42	0.707
LVEDD, mm	49 ± 1.4	49 ± 1.2	>0.99
LVESD, mm	33 ± 0.9	33 ± 1.1	>0.99
LVEF, %	60 ± 1.8	60 ± 1.3	0.742
LV diastolic function			
E-wave, cm/s	60 ± 3.6	61 ± 3.6	0.707
A-wave, cm/s	73 ± 2.8	70 ± 3.5	0.381
E/A ratio	0.8 (0.67 - 1.01)	0.8 (0.72 - 1.08)	0.509 ^a
Structure			
LPVW, mm	9 (8.2 - 9.4)	9 (8.7 - 9.6)	0.340 ^a
LV mass, gram	159 ± 5.9	159 ± 9.9	>0.99
LV mass index, gram/m ²	73 (70.0 - 80.5)	70 (65.7 - 81.2)	0.635 ^a

Left ventricular systolic and diastolic function measured at day 29 of the placebo and resveratrol intervention (n=12). Data are presented as mean /rpm SEM when normally distributed, otherwise median and range are shown. ^aP-value relates to nonparametric Wilcoxon Signed Rank test. HR, heart rate; SV, stroke volume; CO, cardiac output; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; LVEF, left ventricular ejection fraction; LPVW, left ventricular posterior wall thickness; LV mass, Left ventricular mass.

Chapter 7

General discussion

Worldwide the prevalence of individuals diagnosed with type 2 diabetes (T2DM) is increasing rapidly, reaching pandemic proportions (1). Metabolic inflexibility, the decreased ability to switch from fat oxidation in the fasted state to glucose oxidation in the insulin stimulated state, and insulin resistance, are characteristic for type 2 diabetes patients. These impairments in metabolic flexibility and insulin sensitivity are not only present in patients with type 2 diabetes but also in a pre-diabetic state of impaired glucose tolerance (2). Recently, acetylcarnitine has been identified in animal models as an important metabolite in maintaining metabolic flexibility, and subsequently glucose homeostasis and insulin sensitivity (3-6). The enzyme carnitine acetyltransferase (CrAT) catalyzes the formation of acetylcarnitine by conjugating acetyl-CoA and free carnitine (3-6). Formation of acetylcarnitine might prevent accumulation of intra-mitochondrial acetyl-CoA and subsequently may beneficially affect metabolic flexibility and glucose homeostasis. However, in humans, it is still elusive whether similar mechanisms are in place. Therefore, the research in this PhD thesis focused on the role of acetylcarnitine in relation to metabolic flexibility and insulin sensitivity in humans.

How to assess acetylcarnitine concentrations?

Our knowledge about the role of carnitine metabolism and specifically, acetylcarnitine formation in metabolic flexibility and insulin sensitivity in humans is currently limited. One of the reasons is that, until recently, the only means to quantify acetylcarnitine was the determination by mass spectrometry in muscle biopsies (7). The invasiveness of the muscle biopsy is an evident limitation of this method and does not allow dynamic determination of acetylcarnitine concentrations in humans. Therefore, noninvasive alternatives are necessary to assess acetylcarnitine concentration in humans and gain understanding of the role of acetylcarnitine in glucose tolerance, metabolic flexibility and insulin sensitivity. Recently, a proton magnetic resonance spectroscopy (^1H -MRS) technique using long echo times was developed to determine acetylcarnitine concentrations in humans *in vivo* (8). ^1H -MRS is an excellent tool to non-invasively and dynamically assess acetylcarnitine concentrations.

In **chapter 4** we applied this novel ^1H -MRS technique to determine acetylcarnitine concentrations in humans with different glucose homeostasis, i.e. NGT and IGT individuals. Since impairments in metabolic flexibility and insulin sensitivity are not only present in patients with T2DM but also in a pre-diabetic state of impaired glucose tolerance (IGT) (2), the comparison of free carnitine availability and acetylcarnitine

concentrations in individuals with IGT and normal glucose tolerant (NGT) control individuals is interesting. The possibility to perform dynamic acetylcarnitine measurements was instrumental in gaining more detailed knowledge concerning carnitine metabolism in these individuals prone to develop T2DM. This non-invasive MR approach enabled resting acetylcarnitine determination at different time points during the day (i.e. morning 7:00 AM and afternoon 5:00 PM) as well as before exercise and at near-maximal acetylcarnitine abundance, immediately after exercise. Although we found that acetylcarnitine concentrations were comparable between normal and impaired glucose tolerant individuals after an overnight fast, striking differences in morning-afternoon variation were monitored, with the increase in acetylcarnitine levels over the day being blunted in IGT individuals. This highlights the importance of non-invasive tools for repeated measurements and suggests that timing of the acetylcarnitine determination is highly important. In the morning, after an overnight fast, formation of acetylcarnitine is low and therefore this may not be a good time to detect physiologically relevant differences. After an overnight fast, fat will be the main substrate source of the human body and the conversion from acetyl-CoA to acetylcarnitine will be of less importance. Therefore, the determination of acetylcarnitine concentration may be more relevant in a metabolic situation where the conversion from acetyl-CoA to acetylcarnitine is increased. Two time points are insufficient to state the optimal timing for acetylcarnitine determinations and it neither reveals whether acetylcarnitine concentrations have a day-night rhythm. Future research, including more time points for acetylcarnitine determination is needed in order to investigate whether a day-night rhythm is present, to describe a possible rhythm in more detail and to determine optimal timing for determination of acetylcarnitine concentrations.

Furthermore, the fact that with using MRS we can perform dynamic measurements, allows studying of exercise induced changes in acetylcarnitine. Exercise is known to increase skeletal muscle acetylcarnitine. Quite likely, this is because exercise increases substrate load into the mitochondria very rapidly, resulting in increased acetyl-CoA levels. At high exercise intensity, this increase in acetylcarnitine reflects the near-maximal capacity individuals have to produce acetylcarnitine and it can be seen as a parameter for free carnitine availability (5). We determined pre- and post-exercise acetylcarnitine concentration *in vivo* and indeed confirmed that exercise is a potent stimulator of acetylcarnitine formation. The application of this new *in vivo* ^1H -MRS technique worked perfectly in young healthy lean individuals, but in overweight

individuals we noticed that lipid resonances are present in some individuals around the same frequency as the acetylcarnitine peak (2.13Hz). This makes the determination of the isolated acetylcarnitine resonance difficult and lipid contamination of the results is very likely. One way to circumvent this, is to prolong the echo time even further, in order to relatively suppress the lipid resonances even more. Therefore, acetylcarnitine concentration was analyzed in spectra with TE=500 ms in overweight participants in **chapter 4**. However, these long echo times have a large impact on the MR acquisition time, making the measurements lengthy and strongly T2-dependant. Therefore, alternative ways to suppress lipid resonances are needed. This lipid contamination only occurred in overweight individuals with high amounts of adipose tissue infiltration into skeletal muscle. However, since 80% of all people with disturbed glucose homeostasis are overweight (9), it is very important to develop alternative MR sequences preventing this lipid contamination. Therefore, in **chapter 5** we developed an editing sequence to determine acetylcarnitine concentrations with additional lipid suppression. This new alternative sequence makes use of the difference in T1 between acetylcarnitine- and lipid resonances and allows better differentiation between lipids and acetylcarnitine (10). This allows more accurate quantification of acetylcarnitine concentrations at TE=350ms in overweight population in future studies.

Is carnitine availability and acetylcarnitine formation important for metabolic flexibility and insulin sensitivity?

The enzyme carnitine acetyltransferase (CrAT) catalyzes the formation of acetylcarnitine by conjugating acetyl-CoA and free carnitine (3-6). Therefore, skeletal muscle free carnitine availability is a crucial factor for acetylcarnitine formation (3, 4, 11). From rodent studies, it has been shown that skeletal muscle free carnitine availability could be reduced due to high-fat feeding when compared to low-fat feeding. This reduction in free carnitine availability was accompanied by metabolic inflexibility and insulin resistance in these rodents (4, 12-14). In accordance, skeletal muscle free carnitine availability as well as acetylcarnitine were decreased in metabolic inflexible and insulin resistant BAP-agouti transgenic mice (a mouse model for diabetes) compared to control mice (11). These animal data indicate a link between metabolic inflexibility/insulin resistance and low free carnitine availability.

In line with this, human research has recently indicated that high *in vivo* determined acetylcarnitine concentrations are associated with enhanced insulin sensitivity (8),

reporting the lowest acetylcarnitine concentrations in patients with T2DM who are characterized by compromised metabolic flexibility and insulin sensitivity. As acetylcarnitine concentrations in the resting state are correlated with free carnitine availability (3), these data are in line with the hypothesis that low carnitine availability may be causally related to insulin resistance and diabetes. As discussed before, in **chapter 4** we show that individuals with IGT have compromised metabolic flexibility and insulin sensitivity compared to NGT, as reported before (2). *In vivo* assessment of acetylcarnitine concentrations via ^1H -MRS revealed blunted acetylcarnitine concentration in IGT individuals in the afternoon (5:00 PM) and compromised morning-afternoon variation. These data suggest that the capacity to form acetylcarnitine might not only be very important in maintaining metabolic flexibility and insulin sensitivity in animals but also in humans.

Furthermore, we showed in **chapter 4** that maximal acetylcarnitine formation upon exercise was reduced in IGT compared to NGT individuals, together with impaired glucose homeostasis, metabolic inflexibility and insulin resistance. This indicates that an impaired capacity to form acetylcarnitine, possibly caused by a decreased free carnitine availability may be underlying these metabolic derangements and hints at an important role of free carnitine availability in metabolic health.

In contrast to the reduced metabolic flexibility and free carnitine availability with IGT, we showed in **chapter 2** that 3-weeks on an isocaloric high-fat diet did reduce metabolic flexibility despite unchanged skeletal muscle free carnitine availability, acetylcarnitine or other acylcarnitine species assessed after an overnight fast. While these results may question whether free carnitine availability indeed is crucial in high-fat diet induced changes in metabolic flexibility and insulin sensitivity, it should be noted that in fact in this biopsy-based study we cannot conclude anything about the capacity to form acetylcarnitine. Therefore, timing of the measurement may have influenced the results since free carnitine availability, acetylcarnitine and other acylcarnitine species were only determined after an overnight fast. It would be interesting to investigate afternoon concentration and changes over the day in response to a 3-week isocaloric diet to see whether these morning-evening variations could explain the reduction in metabolic flexibility.

Interestingly, although timing might not have been optimal, we found a correlation between free carnitine availability prior to the start of the 3-weeks isocaloric diet and the metabolic response to the diet. Individuals with initially low skeletal muscle free carnitine availability showed a stronger decrease in metabolic flexibility and insulin sensitivity. This suggests that an initially high carnitine availability reflects a high capacity to cope with a dietary challenge indicating the importance of free carnitine availability in metabolic health. It would be of great interest to investigate whether different timing or the capacity to form acetylcarnitine would be predictive as well for the response to a 3-weeks of isocaloric high-fat diet.

Taken together, reduced acetylcarnitine formation is not only shown in metabolic inflexible and insulin resistant rodents but also in humans with compromised metabolic health. Based on this association between free carnitine availability and metabolic flexibility and insulin sensitivity, improving free carnitine availability might be a very potent target to improve metabolic flexibility, insulin sensitivity and glucose tolerance in subject with hampered carnitine availability.

Does an increase in carnitine availability result in improved skeletal muscle acetylcarnitine formation and concomitantly increased metabolic flexibility and insulin sensitivity?

Since acetylcarnitine concentrations are reduced with impaired glucose tolerance (**chapter 4**) and T2DM (8), a diminished free carnitine availability may be likely in these individuals. This raises the idea that addition of carnitine via either supplementation or infusion might improve free carnitine availability and therefore might prevent the development of metabolic inflexibility and insulin sensitivity.

Animal models previously showed that in animals characterized by low free carnitine availability and acetylcarnitine concentrations, carnitine supplementation added to the food or drinking water could elevate these levels to normal control values. The increase in free carnitine availability was accompanied by improved metabolic flexibility and increased insulin sensitivity (4, 11). Whether carnitine supplementation is also able to improve metabolic flexibility in humans still remained elusive. Moreover, although some beneficial effects of carnitine supplementation are reported, it had so far not been studied whether the capacity to form acetylcarnitine is underlying these effects on metabolic flexibility and concomitantly improved glucose tolerance in humans.

We hypothesized that carnitine supplementation in IGT individuals could increase free carnitine availability and subsequently acetylcarnitine formation leading to improved metabolic flexibility and insulin sensitivity. In **chapter 4**, we indeed showed that carnitine supplementation is able to increase skeletal muscle acetylcarnitine concentration in IGT towards NGT control values (in the afternoon). Furthermore, the morning-afternoon variation in acetylcarnitine as well as the capacity to form acetylcarnitine after exercise were restored upon carnitine supplementation in these IGT individuals towards NGT values. So, increasing free carnitine availability improved acetylcarnitine formation in individuals with hampered carnitine availability. In line with previous animal research (4, 11), improving free carnitine availability resulted in restored metabolic flexibility upon carnitine supplementation indicating that acetylcarnitine formation in humans may be underlying beneficial effects on metabolic flexibility. However, contrary to animal research, no alterations in insulin sensitivity were found in IGT after carnitine supplementation. Although the current study cannot reveal the reason for this lack of effect on insulin sensitivity, previous research with carnitine supplementation suggests that the duration of supplementation may have been too short to improve insulin sensitivity (15, 16). Indeed, improvements in fasting plasma glucose and insulin, as well as insulin sensitivity (expressed as HOMA-IR) were reported after oral L-carnitine supplementation for either 12, 24 and 48 weeks. This indicates that the duration of 36 days in our study might be too short to improve insulin sensitivity. Future studies are highly needed to unravel if longer durations of carnitine supplementation could affect insulin sensitivity positively.

A different strategy to increase free carnitine availability is intravenous infusion of carnitine. Previous studies with intravenous carnitine infusion in humans showed positive effects on markers of insulin sensitivity such as glucose infusion rate (GIR) (17, 18) and M-value (19). However, it is still unknown what the underlying mechanism is of these positive effects of intravenous carnitine administration on insulin sensitivity. Therefore, we used lipid infusion as a model of insulin resistance and investigated if carnitine infusion during simultaneous lipid infusion could alleviate lipid-induced insulin resistance and metabolic inflexibility in healthy young males (**chapter 5**). Although lipid infusion resulted in elevated plasma free fatty acid level and decreased metabolic flexibility and insulin resistance as expected (20-25), additional carnitine administration did not result in increased skeletal muscle free carnitine availability or acetylcarnitine formation. The uptake of carnitine into the skeletal muscle via the sodium dependent organic cation

transporter (OCTN2) is facilitated by insulin (26). A possible explanation of the lack of increase in skeletal muscle free carnitine availability upon carnitine infusion may lay in this insulin stimulated carnitine uptake in the muscle. Although insulin concentrations are high, the lipid induced insulin resistance may also affect the OCTN2 receptor, thereby negatively influencing carnitine uptake in skeletal muscle (27). An additional control condition with intravenous infusion of carnitine without additional lipid infusion would be necessary to confirm whether lipid infusion could induce insulin resistance of the OCTN2 receptor. Since we did not perform this, future studies are needed to investigate whether a lipid overload (such as with intravenous lipid infusion) could indeed decrease the insulin-mediated uptake of free carnitine. Therefore, as carnitine availability did not increase in muscle, it is not surprising that metabolic flexibility and insulin sensitivity did not change.

Future perspectives

Supplementation with carnitine in IGT individuals increased plasma and skeletal muscle acetylcarnitine concentrations and concomitantly improved metabolic flexibility (**chapter 4**). In T2DM, acetylcarnitine concentrations are likely to be even more reduced than in IGT individuals. Therefore, an interesting next step would be to investigate whether supplementation with carnitine in T2DM patients could elevate the capacity to form acetylcarnitine in the skeletal muscle and subsequently improve metabolic flexibility and insulin sensitivity. Future studies are needed to investigate whether similar results could be obtained in T2DM patients and therefore, whether carnitine might be a useful add-on therapy to improve glucose tolerance and metabolic health in this patient group. The developed alternative ^1H -MRS sequences will allow investigating of acetylcarnitine concentrations in this typically overweight type 2 diabetes patients (**chapter 5**). We showed in **chapter 4** that carnitine supplementation in IGT individuals resulted in elevated skeletal muscle acetylcarnitine concentrations and concomitantly improved metabolic flexibility. Our results are in line with other studies reporting positive effects of oral L-carnitine supplementation on different metabolic parameters (33-36). However, there are also studies that did not find any effect of oral L-carnitine supplementation in metabolically compromised individuals (15, 16, 36, 37). Hardly any of these studies using L-carnitine reported results on free carnitine availability. A possible explanation for the variable results might be that not every individual responds in the same way and that the individual response to carnitine supplementation depends on the initial carnitine status. It may be that individuals with low carnitine status benefit to a greater extend

from oral L-carnitine supplementation compared to individuals with a normal carnitine status. This may to some extent explain the inconclusive results reported so far. Future studies should address whether difference in initial carnitine status indeed could explain the different results of carnitine on metabolic health.

Furthermore, positive effects of carnitine are not only found on acetylcarnitine, metabolic flexibility and insulin sensitivity but also on physical performance, cognitive function and mental health (28-31). Therefore, it would be very interesting to know whether these positive changes in skeletal muscle acetylcarnitine concentrations and metabolic flexibility also improve overall well-being of the participants. Assessment of sleep, quality of life, cognition, daily activities and general physical performance would be potential targets to investigate the effects of carnitine on general well-being in future studies.

A very interesting finding in this thesis that requires more investigation was the morning-afternoon increase in acetylcarnitine concentrations in NGT individuals and even more striking the hampered morning-afternoon variation in glucose intolerant individuals (**chapter 4**). Future research needs to point out whether type 2 diabetes individuals show also reduced morning-afternoon variation. Why acetylcarnitine concentrations increase during the day is still poorly understood. In line with this increase in acetylcarnitine over the day, Van Moorsel et al. showed that energy expenditure and mitochondrial function were also higher in the evening compared to morning (32). It would be interesting to investigate whether a 24-hour rhythm for acetylcarnitine exist just like for mitochondrial function, and whether this rhythmicity is blunted in T2DM.

REFERENCES

1. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes care*. 2004;27(5):1047-53.
2. Faerch K, Vaag A. Metabolic inflexibility is a common feature of impaired fasting glycaemia and impaired glucose tolerance. *Acta diabetologica*. 2011;48(4):349-53.
3. Muoio DM, Noland RC, Kovalik JP, Seiler SE, Davies MN, DeBalsi KL, et al. Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. *Cell metabolism*. 2012;15(5):764-77.
4. Noland RC, Koves TR, Seiler SE, Lum H, Lust RM, Ilkayeva O, et al. Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control. *The Journal of biological chemistry*. 2009;284(34):22840-52.
5. Stephens FB, Constantin-Teodosiu D, Greenhaff PL. New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *The Journal of physiology*. 2007;581(Pt 2):431-44.
6. Muoio DM. Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. *Cell*. 2014;159(6):1253-62.
7. van Vlies N, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, et al. Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. *The Biochemical journal*. 2005;387(Pt 1):185-93.
8. Lindeboom L, Nabuurs CI, Hoeks J, Brouwers B, Phielix E, Kooi ME, et al. Long-echo time MR spectroscopy for skeletal muscle acetylcarnitine detection. *The Journal of clinical investigation*. 2014;124(11):4915-25.
9. Mantzoros CS. *Obesity and Diabetes*. Totowa: Humana Press; 2006.
10. Lindeboom L, Bruls YM, van Ewijk PA, Hesselink MK, Wildberger JE, Schrauwen P, et al. Longitudinal relaxation time editing for acetylcarnitine detection with 1 H-MRS. *Magnetic resonance in medicine*. 2016.
11. Power RA, Hulver MW, Zhang JY, Dubois J, Marchand RM, Ilkayeva O, et al. Carnitine revisited: potential use as adjunctive treatment in diabetes. *Diabetologia*. 2007;50(4):824-32.
12. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell metabolism*. 2008;7(1):45-56.

13. Wessels B, van den Broek NM, Ciapaite J, Houten SM, Wanders RJ, Nicolay K, et al. Carnitine supplementation in high-fat diet-fed rats does not ameliorate lipid-induced skeletal muscle mitochondrial dysfunction in vivo. *American journal of physiology Endocrinology and metabolism*. 2015;309(7):E670-8.
14. Wang ZQ, Zhang XH, Yu Y, Poulev A, Ribnicky D, Floyd ZE, et al. Bioactives from bitter melon enhance insulin signaling and modulate acyl carnitine content in skeletal muscle in high-fat diet-fed mice. *The Journal of nutritional biochemistry*. 2011;22(11):1064-73.
15. Gonzalez-Ortiz M, Hernandez-Gonzalez SO, Hernandez-Salazar E, Martinez-Abundis E. Effect of oral L-carnitine administration on insulin sensitivity and lipid profile in type 2 diabetes mellitus patients. *Annals of nutrition & metabolism*. 2008;52(4):335-8.
16. Galloway SD, Craig TP, Cleland SJ. Effects of oral L-carnitine supplementation on insulin sensitivity indices in response to glucose feeding in lean and overweight/obese males. *Amino acids*. 2011;41(2):507-15.
17. Capaldo B, Napoli R, Di Bonito P, Albano G, Sacca L. Carnitine improves peripheral glucose disposal in non-insulin-dependent diabetic patients. *Diabetes research and clinical practice*. 1991;14(3):191-5.
18. Mingrone G, Greco AV, Capristo E, Benedetti G, Giancaterini A, De Gaetano A, et al. L-carnitine improves glucose disposal in type 2 diabetic patients. *Journal of the American College of Nutrition*. 1999;18(1):77-82.
19. Giancaterini A, De Gaetano A, Mingrone G, Gniuli D, Liverani E, Capristo E, et al. Acetyl-L-carnitine infusion increases glucose disposal in type 2 diabetic patients. *Metabolism: clinical and experimental*. 2000;49(6):704-8.
20. Boden G, Jadali F, White J, Liang Y, Mozzoli M, Chen X, et al. Effects of fat on insulin-stimulated carbohydrate metabolism in normal men. *The Journal of clinical investigation*. 1991;88(3):960-6.
21. Hoeks J, Hesselink MK, Russell AP, Mensink M, Saris WH, Mensink RP, et al. Peroxisome proliferator-activated receptor-gamma coactivator-1 and insulin resistance: acute effect of fatty acids. *Diabetologia*. 2006;49(10):2419-26.
22. Hoeks J, Mensink M, Hesselink MK, Ekroos K, Schrauwen P. Long- and medium-chain fatty acids induce insulin resistance to a similar extent in humans despite marked differences in muscle fat accumulation. *The Journal of clinical endocrinology and metabolism*. 2012;97(1):208-16.
23. Meex RC, Phielix E, Moonen-Kornips E, Schrauwen P, Hesselink MK. Stimulation of human whole-body energy expenditure by salsalate is fueled by higher lipid oxidation under fasting

- conditions and by higher oxidative glucose disposal under insulin-stimulated conditions. *The Journal of clinical endocrinology and metabolism*. 2011;96(5):1415-23.
24. Phielix E, Meex R, Ouwens DM, Sparks L, Hoeks J, Schaart G, et al. High oxidative capacity due to chronic exercise training attenuates lipid-induced insulin resistance. *Diabetes*. 2012;61(10):2472-8.
 25. Tsintzas K, Chokkalingam K, Jewell K, Norton L, Macdonald IA, Constantin-Teodosiu D. Elevated free fatty acids attenuate the insulin-induced suppression of PDK4 gene expression in human skeletal muscle: potential role of intramuscular long-chain acyl-coenzyme A. *The Journal of clinical endocrinology and metabolism*. 2007;92(10):3967-72.
 26. Stephens FB, Constantin-Teodosiu D, Laithwaite D, Simpson EJ, Greenhaff PL. Insulin stimulates L-carnitine accumulation in human skeletal muscle. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2006;20(2):377-9.
 27. Deachapunya C, Palmer-Densmore M, O'Grady SM. Insulin stimulates transepithelial sodium transport by activation of a protein phosphatase that increases Na-K ATPase activity in endometrial epithelial cells. *The Journal of general physiology*. 1999;114(4):561-74.
 28. Chen T, Li J, Liu J, Li N, Wang S, Liu H, et al. Activation of SIRT3 by resveratrol ameliorates cardiac fibrosis and improves cardiac function via the TGF-beta/Smad3 pathway. *American journal of physiology Heart and circulatory physiology*. 2015;308(5):H424-34.
 29. Jain S, Singh SN. Effect of L-carnitine Supplementation on Nutritional Status and Physical Performance Under Calorie Restriction. *Indian journal of clinical biochemistry : IJCB*. 2015;30(2):187-93.
 30. Jing E, Emanuelli B, Hirschey MD, Boucher J, Lee KY, Lombard D, et al. Sirtuin-3 (Sirt3) regulates skeletal muscle metabolism and insulin signaling via altered mitochondrial oxidation and reactive oxygen species production. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108(35):14608-13.
 31. Samir SM, Abbas AM, Safwat SM, Elserougy HG. Effect of L-carnitine on diabetes-induced changes of skeletal muscles in rats. *Journal of basic and clinical physiology and pharmacology*. 2017.
 32. van Moorsel D, Hansen J, Havekes B, Scheer FA, Jorgensen JA, Hoeks J, et al. Demonstration of a day-night rhythm in human skeletal muscle oxidative capacity. *Molecular metabolism*. 2016;5(8):635-45.
 33. Malaguarnera M, Gargante MP, Russo C, Antic T, Vacante M, Malaguarnera M, et al. L-carnitine supplementation to diet: a new tool in treatment of nonalcoholic

- steatohepatitis—a randomized and controlled clinical trial. *The American journal of gastroenterology*. 2010;105(6):1338-45.
34. Molino A, Cascino A, Conte C, Ramaccini C, Rossi Fanelli F, Laviano A. Caloric restriction and L-carnitine administration improves insulin sensitivity in patients with impaired glucose metabolism. *JPEN Journal of parenteral and enteral nutrition*. 2010;34(3):295-9.
 35. Rahbar AR, Shakerhosseini R, Saadat N, Taleban F, Pordal A, Goldestan B. Effect of L-carnitine on plasma glycemic and lipidemic profile in patients with type II diabetes mellitus. *European journal of clinical nutrition*. 2005;59(4):592-6.
 36. Ringseis R, Keller J, Eder K. Role of carnitine in the regulation of glucose homeostasis and insulin sensitivity: evidence from in vivo and in vitro studies with carnitine supplementation and carnitine deficiency. *European journal of nutrition*. 2012;51(1):1-18.
 37. Bloomer RJ, Fisher-Wellman KH, Tucker PS. Effect of oral acetyl L-carnitine arginate on resting and postprandial blood biomarkers in pre-diabetics. *Nutrition & metabolism*. 2009;6:25.

The background is a vibrant teal color. In the upper left, there are stylized, overlapping geometric shapes in red and yellow. A large, stylized figure in a red and yellow swimsuit is depicted in a diving pose, falling from a dark blue mountain peak. The mountain has two vertical red lines on its summit, each topped with a yellow circle. A large, textured yellow sphere is positioned near the diver. To the right, a red circle with a horizontal line through it is visible. The lower half of the image is a deep blue gradient, with large, stylized red petals or leaves at the bottom corners. A cloud of white particles is scattered around the base of the mountain.

Addendum

Summary

Samenvatting

Valorization

Dankwoord

List of publications

About the author

SUMMARY

The number of people with overweight is increasing rapidly worldwide, reaching pandemic proportions. In 2014, 39% of the adults were overweight and 13% were obese. A combination of excessive food intake (high-caloric, high-fat) and low physical activity are the primary contributors to the development of overweight (1). Overweight is associated with the development of chronic metabolic disease such as type 2 diabetes mellitus (T2DM). An important characteristic of T2DM is that the metabolic effects of insulin on glucose metabolism are blunted (2). As the prevalence of T2DM is tightly linked to obesity, also T2DM, just like overweight, is increasing dramatically worldwide. Recent estimates reported 171 million people worldwide diagnosed with T2DM in the year 2000 and expected to increase towards 366 million in 2030 (3). Since T2DM is associated with reduced quality of life, decreased life expectancy, and increased risk of morbidities such as cardiovascular diseases, the diabetes-related costs are a major burden on our health care systems. Therefore, it is important to increase our understanding of this disease to improve prevention and cure.

Decreased metabolic flexibility is an early hallmark in the development of T2DM. Acetylcarnitine has recently been suggested as a very important metabolite in maintaining metabolic flexibility, and subsequently glucose homeostasis and insulin sensitivity (4-7). Free carnitine availability is identified as being crucial in acetylcarnitine formation and maintaining metabolic flexibility (4, 5, 8).

In **chapter 2** we investigated the role of acylcarnitines in a human intervention that is expected to modify substrate metabolism and metabolic flexibility. The effects of three weeks isoenergetic high-fat feeding on metabolic flexibility and insulin sensitivity in overweight male volunteers are described. High fat diet is known to decrease metabolic flexibility, and we therefore investigated if changes in free carnitine availability, acetylcarnitine, or other carnitine species could explain the earlier reported decrease in metabolic flexibility. Acylcarnitines and free carnitine were unchanged in response to the diet. However, we revealed that the muscle concentration of free carnitine was related to the metabolic response with the diet and therefore, volunteers with low free carnitine availability at the beginning of the dietary intervention showed the most pronounced decrease in insulin sensitivity and metabolic flexibility suggesting free carnitine availability as a predictive marker for metabolic response to a change in diet.

This gave rise to the hypothesis that enhancing free carnitine in metabolic compromised humans might be a good strategy to combat metabolic inflexibility and insulin resistance by increasing the capacity to form acetylcarnitine. Possible strategies to enhance free carnitine availability could be intravenous infusion or oral supplementation of carnitine. In **chapter 3**, we investigated if acute carnitine infusion during simultaneous lipid infusion could alleviate lipid-induced insulin resistance and metabolic inflexibility in healthy young males by increasing free carnitine availability. As expected, the intravenous lipid infusion elevated plasma free fatty acid concentration and resulted in a hampered insulin sensitivity and metabolic flexibility in healthy, insulin sensitive volunteers. Intravenous infusion of carnitine in addition to lipid elevated plasma free carnitine availability. However, against expectations, carnitine infusion did not increase skeletal muscle free carnitine availability possibly due to insulin resistance of the OCTN2 receptor involved in skeletal muscle carnitine uptake. In line with the lack of increase in skeletal muscle free carnitine availability, lipid-induced metabolic inflexibility and insulin resistance was not rescued by carnitine administration.

Next to the short-term elevation in carnitine availability by intravenous infusions, we also aimed to investigate the longer term effect upon a five-week intervention with oral carnitine supplementation. Therefore, **chapter 4** describes the results of a double-blind, randomized placebo-controlled trial investigating if oral carnitine supplementation improves metabolic flexibility and insulin resistance in volunteers with impaired glucose tolerance. Furthermore, it was investigated whether this is mediated by increased formation of acetylcarnitine. Carnitine supplementation had very pronounced effects on metabolic flexibility in impaired glucose tolerant volunteers, and in fact was able to completely restore metabolic flexibility. Carnitine supplementation enhanced the increase in acetylcarnitine concentration in resting muscle over the day as well as the capacity to form acetylcarnitine with exercise. These changes in acetylcarnitine formation may be underlying the beneficial effects on metabolic flexibility. Taken together, carnitine supplementation may be an interesting aid in improving disturbed metabolism in subjects prone to develop type 2 diabetes mellitus.

In **chapter 5**, we developed an alternative Magnetic Resonance Spectroscopy (MRS) sequence to detect acetylcarnitine concentration in vivo. Previously, we reported that acetylcarnitine can be detected by MRS, using long echo times ($TE=350$ ms) as lipid resonances are suppressed due to short T_2 relaxation. In chapter 3 and 4 we applied this

MR technique. However, the spectra with TE=350 ms that were analyzed in chapter 4 still showed considerable lipid contamination in overweight participants due to strong adipose tissue marbling of muscle in these subjects (and therefore, even longer echo times were used for acetylcarnitine quantification in chapter 4). Thus, more pronounced T₂-weighting proved necessary in this typical overweight population to sufficiently suppress adipose tissue signal and determine acetylcarnitine. Therefore, a sequence that also makes use of the difference in T₁ between acetylcarnitine- and lipid resonances was developed. This new (alternative) methodology allows to better suppress the lipid signal, which is especially important in subjects with high abundance of intramuscular fat. This allows more accurate quantification of acetylcarnitine concentrations at TE=350ms in this population in future studies.

In **chapter 6**, we investigated the role of acylcarnitines in a human intervention with resveratrol. Resveratrol has been shown to improve mitochondrial function and metabolic health in obese humans, and provides another model to investigate the relationship between acylcarnitines and metabolic flexibility in humans. The effects of the food supplement resveratrol on insulin sensitivity, metabolic flexibility, mitochondrial function and skeletal muscle acylcarnitine species in impaired glucose tolerant subjects were investigated but while mitochondrial function was stimulated in response to resveratrol, no effects of resveratrol were found on other metabolic health parameters and in line, acylcarnitine concentrations remained unchanged.

While up to now, reduced acetylcarnitine formation was only reported in metabolic inflexible and insulin resistant rodents. This thesis shows that the formation of acetylcarnitine is also reduced in humans with compromised metabolic health. Based on this association between free carnitine availability and metabolic flexibility and insulin sensitivity, improving free carnitine availability might be a very potent target to improve metabolic flexibility, insulin sensitivity and glucose tolerance in subject with hampered carnitine availability. Although lipid-induced metabolic inflexibility and insulin resistance could not be rescued by carnitine administration, supplementation with carnitine had very pronounced effects of on metabolic flexibility in impaired glucose tolerant volunteers, and in fact can completely restore metabolic flexibility. Therefore, carnitine supplementation may be an interesting aid in improving disturbed metabolism in subjects prone to develop type 2 diabetes mellitus.

REFERENCES

1. Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128.9 million children, adolescents, and adults. *Lancet* (London, England). 2017.
2. Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes*. 2000;49(5):677-83.
3. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes care*. 2004;27(5):1047-53.
4. Muoio DM, Noland RC, Kovalik JP, Seiler SE, Davies MN, DeBalsi KL, et al. Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. *Cell metabolism*. 2012;15(5):764-77.
5. Noland RC, Koves TR, Seiler SE, Lum H, Lust RM, Ilkayeva O, et al. Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control. *The Journal of biological chemistry*. 2009;284(34):22840-52.
6. Stephens FB, Constantin-Teodosiu D, Greenhaff PL. New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *The Journal of physiology*. 2007;581(Pt 2):431-44.
7. Muoio DM. Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. *Cell*. 2014;159(6):1253-62.
8. Power RA, Hulver MW, Zhang JY, Dubois J, Marchand RM, Ilkayeva O, et al. Carnitine revisited: potential use as adjunctive treatment in diabetes. *Diabetologia*. 2007;50(4):824-32.

SAMENVATTING

Het aantal mensen met overgewicht neemt wereldwijd snel toe en bereikt pandemische proporties. In 2014 had 39% van de volwassenen overgewicht en was 13% zwaarlijvig. Een combinatie van overmatige voedselinname (hoog-calorisch, hoog vetgehalte) en weinig lichaamsbeweging zijn de belangrijkste oorzaken van de ontwikkeling van overgewicht (1). Overgewicht is geassocieerd met de ontwikkeling van chronische metabole ziekten zoals diabetes mellitus type 2 (T2DM). Een belangrijk kenmerk van T2DM is dat de metabole effecten van insuline op het glucose metabolisme verminderd zijn (2). Aangezien de prevalentie van T2DM nauw verbonden is met obesitas, neemt ook T2DM, net als overgewicht, wereldwijd dramatisch toe. Volgens recente schattingen zijn in de loop van het jaar 2000, 171 miljoen mensen wereldwijd gediagnosticeerd met T2DM en zal dit naar verwachting toenemen tot 366 miljoen in 2030 (3). Omdat T2DM geassocieerd is met een verminderde kwaliteit van leven, een lagere levensverwachting en een verhoogd risico op morbiditeit, zoals hart- en vaatziekten, zijn de diabetes gerelateerde kosten een grote belasting voor ons zorgstelsel. Daarom is het belangrijk om ons inzicht in deze ziekte te vergroten om preventie en genezing te verbeteren.

Verminderde metabole flexibiliteit is een vroeg kenmerk in de ontwikkeling van T2DM. Acetylcarnitine is onlangs gesuggereerd als een zeer belangrijke metaboliet voor het behoud van de metabole flexibiliteit, hetgeen leidt tot betere glucose homeostase en insulinegevoeligheid (4-7). Vrije beschikbaarheid van carnitine wordt beschouwd als cruciaal voor de vorming van acetylcarnitine en het behoud van metabole flexibiliteit (4, 5, 8).

In **hoofdstuk 2** onderzochten we de rol van acylcarnitines in een menselijke interventie waarvan wordt verwacht dat deze het substraatmetabolisme en metabole flexibiliteit zal veranderen. De effecten van drie weken iso-energetisch hoog vetrijk dieet op metabole flexibiliteit en insuline gevoeligheid bij mannelijke vrijwilligers met overgewicht worden beschreven. Van een vetrijk dieet is bekend dat het de metabole flexibiliteit vermindert en daarom hebben we onderzocht of veranderingen in de beschikbaarheid van vrij beschikbaar carnitine, acetylcarnitine of andere carnitine soorten de eerder benoemde vermindering in metabole flexibiliteit kan verklaren. Acylcarnitines en vrije beschikbaar carnitine waren onveranderd door het dieet. Echter, we hebben aangetoond dat de spierconcentratie van vrij carnitine gerelateerd was aan de metabole respons van het

dieet, gezien vrijwilligers met een lage vrije beschikbaarheid van carnitine aan het begin van de dieetinterventie de meest uitgesproken afname in insulinegevoeligheid en metabole flexibiliteit toonden. Dit duidt erop dat carnitine beschikbaarheid een voorspellende marker is voor de metabole respons op een verandering in het dieet.

Dit resulteerde in de hypothese dat het verhogen van vrij beschikbaar carnitine in metabool gecompromitteerde mensen een goede strategie kan zijn om metabole inflexibiliteit en insuline resistentie tegen te gaan door de vorming van acetylcarnitine te vergroten. Mogelijke strategieën om de beschikbaarheid van vrij carnitine te verbeteren, kunnen intraveneuze infusie of orale suppletie van carnitine zijn.

In **hoofdstuk 3** hebben we onderzocht of acute carnitine-infusie tijdens simultane infusie van lipiden de door lipiden genduceerde insuline resistentie en metabole inflexibiliteit bij gezonde jonge mannen kon verminderen door de beschikbaarheid van vrij beschikbaar carnitine te verhogen. De intraveneuze lipiden infusie verhoogde de vrije vetzuurconcentratie in het plasma en resulteerde in een verminderde metabole flexibiliteit en insulinegevoeligheid bij gezonde, insuline-gevoelige vrijwilligers. Intraveneuze infusie van carnitine naast infusie van lipiden verhoogde de vrije carnitine beschikbaarheid in het plasma. Echter, tegen de verwachting in, verhoogde infusie van carnitine de beschikbaarheid van vrij carnitine in de skeletspier niet. Dit is mogelijk het gevolg van insuline resistentie van de OCTN2-receptor die betrokken is bij de opname van carnitine in de skeletspieren. In overeenstemming met de afwezigheid in toename van vrij carnitine in de skeletspier, kon de door lipiden genduceerde metabole inflexibiliteit en insuline resistentie niet worden tegengegaan door intraveneuze toediening van carnitine.

Naast de acute toediening van carnitine, hebben we het effect op langere termijn onderzocht tijdens een interventie van vijf weken met orale suppletie met carnitine.

Hoofdstuk 4 beschrijft de resultaten van deze dubbelblinde, gerandomiseerde, placebo-gecontroleerde studie waarin werd onderzocht of orale suppletie met carnitine de metabole flexibiliteit en insuline resistentie verbeterde bij vrijwilligers met een gestoorde glucosetolerantie. Daarnaast werd onderzocht of dit wordt gemedieerd door een verhoogde vorming van acetylcarnitine. Carnitine suppletie had zeer uitgesproken effecten met betrekking tot metabole flexibiliteit bij gestoorde glucosetolerante vrijwilligers, immers het kan de metabole flexibiliteit volledig herstellen. Suppletie met carnitine versterkte daarnaast de toename van de acetylcarnitine-concentratie in de

skeletspier in rustsituaties gedurende de dag, evenals de capaciteit om acetylcarnitine te vormen bij inspanning. Deze veranderingen in acetylcarnitine-vorming kunnen ten grondslag liggen aan de gunstige effecten op de metabole flexibiliteit. Samengenomen kan carnitine suppletie een interessant hulpmiddel zijn bij het verbeteren van het verstoorde metabolisme bij personen die vatbaar zijn voor het ontwikkelen van diabetes mellitus type 2.

In **hoofdstuk 5** hebben we een alternatieve Magnetische Resonantie Spectroscopie (MRS) -sequentie ontwikkeld om de acetylcarnitine-concentratie *in vivo* te detecteren. Eerder rapporteerden we dat acetylcarnitine kan worden gedetecteerd door MRS, met behulp van lange echotijden ($TE=350$ ms), omdat lipiden-resonanties worden onderdrukt als gevolg van korte T_2 relaxatie. In **hoofdstuk 3** en **4** hebben we deze MR-techniek toegepast. Echter, de spectra met $TE=350$ ms die in **hoofdstuk 4** werden geanalyseerd, vertoonden nog altijd aanzienlijke lipidencontaminatie bij deelnemers met overgewicht als gevolg van vetweefsel contaminatie (en daarom werden in **hoofdstuk 4** zelfs langere echotijden gebruikt voor de kwantificatie van acetylcarnitine). Derhalve bleek een meer uitgesproken T_2 -weging in deze typische populatie met overgewicht noodzakelijk om vetweefsel signaal voldoende te onderdrukken en acetylcarnitine concentraties te bepalen. Daarom werd een sequentie ontwikkeld die ook gebruik maakt van het verschil in T_1 tussen acetylcarnitine- en lipiden-resonanties. Deze nieuwe (alternatieve) methode maakt het mogelijk om het lipiden signaal beter te onderdrukken, wat vooral belangrijk is in personen met een hoge hoeveelheid intramusculair vet. Dit maakt een nauwkeurigere kwantificering van acetylcarnitine-concentraties bij $TE=350$ ms in deze populatie mogelijk in toekomstig onderzoek.

In **hoofdstuk 6** hebben we de rol van acylcarnitines in een menselijke interventie met resveratrol onderzocht. Van resveratrol is aangetoond dat het de mitochondriale functie en metabole gezondheid verbetert bij mensen met obesitas. Hierdoor kan het gebruikt worden als model om de relatie tussen acylcarnitines en metabole flexibiliteit bij mensen te onderzoeken. De effecten van het voedingssupplement resveratrol op insulinegevoeligheid, metabole flexibiliteit, mitochondriale functie en skeletspier acylcarnitine soorten bij gestoorde glucosetolerante personen werden onderzocht. Hoewel de mitochondriale functie werd gestimuleerd door resveratrol, werden er geen effecten van resveratrol gevonden op andere metabole gezondheidsparameters en in lijn hiermee bleven de acylcarnitine concentraties onveranderd.

Tot nu toe werd een verminderde vorming van acetylcarnitine alleen gerapporteerd in metabool inflexibele en insuline resistente knaagdieren. Dit proefschrift laat zien dat de vorming van acetylcarnitine ook wordt verminderd bij mensen met een verminderde metabole gezondheid. Op basis van deze associatie tussen de beschikbaarheid van vrije carnitine en metabole flexibiliteit en insulinegevoeligheid, kan het verbeteren van de beschikbaarheid van vrij carnitine een zeer krachtig doelwit zijn om de metabole flexibiliteit, insulinegevoeligheid en glucosetolerantie bij de proefpersonen te verbeteren met een lage carnitine beschikbaarheid. Hoewel door lipiden genduceerde metabole inflexibiliteit en insuline resistentie niet voorkomen werden door simultane toediening van carnitine, had suppletie met carnitine zeer uitgesproken effecten op metabole flexibiliteit bij vrijwilligers met een gestoorde glucosetolerantie. Daarom kan carnitine suppletie een interessant hulpmiddel zijn bij het verbeteren van het verstoorde metabolisme bij personen die vatbaar zijn voor de ontwikkeling van diabetes mellitus type 2.

REFERENTIES

1. Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128.9 million children, adolescents, and adults. *Lancet* (London, England). 2017.
2. Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes*. 2000;49(5):677-83.
3. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes care*. 2004;27(5):1047-53.
4. Muoio DM, Noland RC, Kovalik JP, Seiler SE, Davies MN, DeBalsi KL, et al. Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. *Cell metabolism*. 2012;15(5):764-77.
5. Noland RC, Koves TR, Seiler SE, Lum H, Lust RM, Ilkayeva O, et al. Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control. *The Journal of biological chemistry*. 2009;284(34):22840-52.
6. Stephens FB, Constantin-Teodosiu D, Greenhaff PL. New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *The Journal of physiology*. 2007;581(Pt 2):431-44.
7. Muoio DM. Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. *Cell*. 2014;159(6):1253-62.
8. Power RA, Hulver MW, Zhang JY, Dubois J, Marchand RM, Ilkayeva O, et al. Carnitine revisited: potential use as adjunctive treatment in diabetes. *Diabetologia*. 2007;50(4):824-32.

VALORIZATION

Social relevance

The number of people with overweight is increasing rapidly worldwide, reaching pandemic proportions. In 2014, 39% of the adults were overweight and 13% were obese worldwide. A combination of excessive food intake (high-caloric, high-fat) and low physical activity are the primary contributors to the development of overweight (1). Overweight is associated with the development of chronic metabolic disease such as type 2 diabetes mellitus (T2DM). An important characteristic of T2DM is that the metabolic effects of insulin on glucose metabolism are blunted (2). In the healthy state, the plasma concentration of glucose is tightly regulated within a narrow range. After food intake, the plasma concentration of the hormone insulin raises, causing the uptake of glucose into tissue, thereby regulating plasma glucose levels. In T2DM, this regulation is poor and hyperglycemia can develop. As the prevalence of T2DM is tightly linked to obesity, also T2DM, just like overweight, is increasing dramatically worldwide. Recent estimates reported 171 million people worldwide diagnosed with T2DM in the year 2000 and expected to increase towards 366 million in 2030 (5). Since T2DM is associated with reduced quality of life, decreased life expectancy, and increased risk of morbidities such as cardiovascular diseases, the diabetes-related costs are a major burden on our health care systems. Therefore, it is important to increase our understanding of this disease to improve prevention and cure. Nowadays treatment of T2DM consist of glucose lowering medication, diet, increasing physical activity or a combination of these. However, T2DM is a complex metabolic disease whereby underlying mechanism are yet not completely understood and not all patients respond well to the conventional treatment. Understanding the underlying mechanisms might result in new treatment strategies.

Activities and products

The research performed in this thesis was executed as a tight collaboration of the department of radiology and the department of Nutrition and Movement Sciences at the Maastricht University Medical Center, within the Diabetes and Metabolism Research group (www.dmrp.nl). The Diabetes and Metabolism Research group focusses on unraveling the underlying mechanisms in the etiology of type 2 diabetes Mellitus (T2DM) by performing translation research. In the current thesis we applied *non-invasive* magnetic resonance spectroscopy (MRS) techniques to investigate acetylcarnitine metabolism in healthy and metabolically compromised individuals.

^1H -MRS is an excellent tool to non-invasively and dynamically assess acetylcarnitine concentrations in contrast to muscle biopsies which are invasive and do not allow dynamic determination of metabolites in humans.

The results presented in the current thesis are or will be implemented in original scientific articles. These articles have been published or are submitted to international well-recognized peer-reviewed journals. The articles can be found online and can be assessed by scientist worldwide. In addition, results and knowledge obtained from the studies performed in this thesis have been presented and communicated to the scientific community on national and international conferences via oral presentations and posters. In this way, the international scientific community can take notice of the current results, thereby advancing the knowledge of the field.

Innovation

Our knowledge about the role of carnitine metabolism and specifically, acetylcarnitine formation in metabolic flexibility and insulin sensitivity in humans is currently limited. One of the reasons is that, until recently, the only means to quantify acetylcarnitine was the determination by mass spectrometry in muscle biopsies (7). The invasiveness of the muscle biopsy is an evident limitation of this method and does not allow dynamic determination of acetylcarnitine concentrations in humans. The recently developed proton magnetic resonance spectroscopy (^1H -MRS) technique using long echo times to determine acetylcarnitine concentrations in humans *in vivo* (8) opens a broad window of opportunities to assess acetylcarnitine concentration in humans and gain understanding of the role of acetylcarnitine in glucose tolerance, metabolic flexibility and insulin sensitivity. ^1H -MRS is an excellent tool to non-invasively and dynamically assess these acetylcarnitine concentrations.

In this thesis, we applied this novel ^1H -MRS technique to determine acetylcarnitine concentrations in humans with different glucose homeostasis, normal glucose tolerant overweight (NGT) and impaired glucose tolerant (IGT) individuals. The possibility to perform dynamic acetylcarnitine measurements was instrumental in gaining more detailed knowledge concerning carnitine metabolism in these individuals prone to develop T2DM. This non-invasive MR approach enabled resting acetylcarnitine determination at different time points during the day (i.e. morning 7:00 AM and afternoon 5:00 PM) as well as before exercise and at near-maximal acetylcarnitine

abundance, immediately after exercise, showing striking differences between individuals with different glucose homeostasis.

However, in overweight individuals we noticed that lipid resonances are present in some individuals around the same frequency as the acetylcarnitine peak (2.13Hz), a problem not present in young healthy lean individuals. This makes the determination of the isolated acetylcarnitine resonance difficult and lipid contamination of the results is very likely. One way to circumvent this and applied in the current thesis, is to prolong the echo time even further, in order to relatively suppress the lipid resonances even more. However, these long echo times have a large impact on the MR acquisition time, making the measurements lengthy and strongly T2-dependant. Therefore, we developed an editing sequence to determine acetylcarnitine concentrations with additional lipid suppression. This new alternative sequence makes use of the difference in T1 between acetylcarnitine- and lipid resonances and allows better differentiation between lipids and acetylcarnitine (10). This allows more accurate quantification of acetylcarnitine concentrations at TE=350ms in overweight population, thereby opening a broad window of opportunity for researchers worldwide to investigate the metabolite acetylcarnitine non-invasively and over time in humans.

Target groups

In this thesis we reported that supplementation with carnitine positively affects the formation of acetylcarnitine and improved metabolic flexibility in individuals at risk to develop type 2 diabetes. Therefore, carnitine supplementation might be an interesting aid to postpone or even prevent the development of T2DM in these individuals at risk. This strikes out the great importance of carnitine in this pre-diabetic population. Since carnitine is a free available food supplement, carnitine intake is a feasible and directly applicable strategy to postpone the development of diabetes. The latter could be of importance to pharmaceutical industry to improve the use of the food supplement carnitine.

Although further research is needed to investigate the effect of carnitine in T2 diabetic patients, animal research indicates very promising results already. The current thesis might be a good rationale for further investigations on the potential of carnitine as important add-on therapy to the usual anti-diabetic treatment which might be very important for patients suffering from type 2 diabetes.

Therefore, the role of carnitine in individuals at risk to develop diabetes and type 2 diabetic patients is of great importance to general practitioners, endocrinologist, dieticians and life style coaches. These professions are in direct contact with the patients and individuals at risk and can directly indicate the advantage of carnitine supplementation on glucose metabolism. Personalized treatment to combat diabetes, e.g. different treatment based on high or low carnitine status, might be an important role for these professions to treat type 2 diabetic patients and individuals at risk. A possible personalization of carnitine supplementation based on the novel acetylcarnitine measurements by proton magnetic resonance spectroscopy (with the long TE protocol) would provide an easy, robust tool that can be applied on any clinical MRI scanner.

Planning and realization

Supplementation with carnitine in IGT individuals increased plasma and skeletal muscle acetylcarnitine concentrations and concomitantly improved metabolic flexibility. In T2DM, acetylcarnitine concentrations are likely to be even more reduced than in IGT individuals. Therefore, an interesting next step would be to investigate whether supplementation with carnitine in T2DM patients could elevate the capacity to form acetylcarnitine in the skeletal muscle and subsequently improve metabolic flexibility and insulin sensitivity. Carnitine might be a useful add-on therapy to improve glucose tolerance and metabolic health in this patient group. Furthermore, understanding whether the acetylcarnitine concentration in muscle (as determined by MRS) is a valid predictor of the success of carnitine supplementation in type 2 diabetic patients might be very important in personalized treatment. If this is indeed a valid predictor, tailored supplementation in type 2 diabetes patients to restore insulin sensitivity and metabolic flexibility might be possible. This may open a window of opportunity to screen patients with type 2 diabetes and subject at risk of developing type 2 diabetes (such as impaired glucose tolerant individuals) for skeletal muscle carnitine status. Based on the skeletal muscle carnitine status it could be decided whether carnitine supplementation is beneficial. Just as carnitine may be an interesting add-on therapy in type 2 diabetic patients with low carnitine status, carnitine could also be an important food supplement in populations at risk of developing diabetes. Currently, it is unclear which subgroups of diabetic patients would benefit the most from carnitine supplementation, as the carnitine concentration in plasma (which can be determined easily) is very different from carnitine status in skeletal muscle (which up to now required muscle biopsies). I will continue to perform human studies to investigate the possible opportunity of carnitine as a tailored add on therapy in T2DM patients.

REFERENCES

1. Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128.9 million children, adolescents, and adults. *Lancet* (London, England). 2017.
2. Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes*.
3. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes care*. 2004;27(5):1047-53.
4. van Vlies N, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, et al. Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. *The Biochemical journal*. 2005;387(Pt 1):185-93.
5. Lindeboom L, Nabuurs CI, Hoeks J, Brouwers B, Phielix E, Kooi ME, et al. Long-echo time MR spectroscopy for skeletal muscle acetylcarnitine detection. *The Journal of clinical investigation*. 2014;124(11):4915-25.
6. Lindeboom L, Bruls YM, van Ewijk PA, Hesselink MK, Wildberger JE, Schrauwen P, et al. Longitudinal relaxation time editing for acetylcarnitine detection with 1 H-MRS. *Magnetic resonance in medicine*. 2016.

DANKWOORD

Het is zover, mijn boekjes is af! En reis van vier jaar (en een beetje) met vele hoogte punten is ten einde. Graag wil ik dit laatste hoofdstuk dan ook gebruiken om iedereen die een bijdrage aan mijn proefschrift heeft geleverd te bedanken.

Allereerst mijn promotieteam; Vera, Patrick en Joachim. Ik ben ontzettend blij dat jullie mij de kans hebben gegeven om het onderzoek in te gaan en wil jullie bedanken voor het vertrouwen en alle tijd en moeite die jullie in mij en mijn proefschrift hebben gestoken. Van jullie wetenschappelijk kijk en kritische vragen heb ik heel veel geleerd de afgelopen jaren, dankjewel hiervoor. Matthijs, ondanks dat jij op papier geen deel uitmaakt van mijn promotieteam, ben je voor mij een volwaardig lid van het team geweest! Jouw aanwezigheid tijdens besprekingen en frisse kijk op data hebben een grote bedrage gehad aan mijn proefschrift, bedankt.

Thanks to the members of the assessment committee, Prof. dr. R.P. Mensink, Prof. dr. A.H. Kersten, Prof. dr. S.W.M. Olde Damink, Prof. dr. R Shiri-Sverdlov, Dr. M. Soeters and Prof. dr. M.E. Kooi, to take time and effort to read and review my thesis and/or being present at the official PhD defense. Prof. D. Muoio, thank you for coming over for my defense.

Marlies (of moet ik zeggen queen bitch), ik ben heel blij dat jij mijn paranimf bent. Samen begonnen wij ruim vier jaar geleden aan onze promotietrajecten binnen de SHOCK groep. Inmiddels ben ik de tel kwijt van het aantal clamps, scans en andere testen die we samen gedaan hebben voor jou resveratrol projecten en mijn carnitine studies. Het staat als een huis dat er geen enkele saaie testdag tussen zat! Ontzettend bedankt voor deze fijne en gezellige samenwerking. Nu we beiden nog een tijdje in SHOCK blijven hoop ik dat er nog vele van deze dagen volgen. Niet alleen op het werk, maar ook naast het werk was jij heel belangrijk. Ik heb ontzettend genoten van alle gezellige filmavondjes, uiteten gaan, pinkpop weekendjes en logeerpartijtjes. Succes met de laatste loodjes van het afronden van jouw thesis!!

Raf, mijn allereerste kamergenootje tijdens mijn promotie-avontuur. Jij was degene die mij wegwijs maakte op de uni! Voor al mijn vragen en problemen had jij een antwoord dan wel oplossing klaar. Ondanks dat we nooit samen aan een onderzoek gewerkt

hebben, heb ik veel van jou expertise en kijk op dingen geleerd. Naast het werk zijn wij goede vrienden geworden en ik vond het dan ook een eer om vorig jaar achter jou te staan als paranimf. Binnenkort zullen de rollen omgedraaid zijn en ik ben heel blij dat jij op deze speciale dag dan ook achter mij wilt staan als paranimf.

Uiteraard is alle werk in deze thesis niet alleen door mij tot stand gekomen. In het bijzonder wil ik iedereen bedanken die mij geholpen heeft met het uitvoeren van alle onderzoeken. Op de eerste plaats alle proefpersonen, zonder jullie inzet was dit proefschrift nooit tot stand gekomen. Hartelijk dank voor jullie deelname. Alle (ex)collegas van SHOCK, HB en radiologie, dankjewel voor de goede en gezellige samenwerking al deze jaren.

Yvo, mijn vaste clamp-maatje sinds jij iets meer dan een jaar geleden begonnen bent in SHOCK. Slechts 5 werkdagen kon je genieten voordat een overvol testschema (nota bene beginnend op jou verjaardag) voor je klaarstond. Ondanks deze al vroeg geboren slechte traditie van het samen clampen op onze verjaardagen ben ik benieuwd hoeveel verjaardags-clamps er nog zullen volgen! De teller staat nu op drie. Ontzettend bedankt voor al jou hulp en ik kijk uit naar onze samenwerking in de toekomst.

Gert, Esther K. en Johanna, een speciaal dankjewel voor jullie als technicians. Invriezen van bipten in de vroege ochtend en het verrichten van ontelbare analyses hebben een belangrijke bijdrage geleverd aan dit proefschrift. Dank jullie wel! Een medisch dankjewel voor Dirk en Niels voor het nemen van ontelbare bipten en alle medische hulp tijdens testdagen. Esther P., Lucas en Bas, jullie expertise en hulp op respectievelijk clamp, MR en medisch gebied was van onmisbare waarde bij het uitvoeren van mijn onderzoeken en ik ben dankbaar voor alles wat ik van jullie geleerd heb. Jan, Genevieve, Ellen, Barbora, Rik, Rob, Anne, Carlijn, Kay, bedankt voor alle gezellige lunches, pauze snack momentjes, etentjes, surfvakanties en andere leuke activiteiten!

Roger, mijn "kleine broertje", dankjewel voor alle hulp bij mijn boekje. Mede dankzij jou ligt er nu een heel mooi eindresultaat van vier jaar hard werken. Heel veel succes met je master die je recent begonnen bent! Papa en mama, dat ik zo ver gekomen ben heb ik voor een groot deel aan jullie te danken! Jullie zijn degene die mij altijd steunden en vertrouwen gaven om te doen wat me tot hier gebracht heeft. Dankjewel dat ik altijd op jullie kan rekenen en altijd voor me klaar staan.

LIST OP PUBLICATIONS

1. **Bruls YMH**, de Ligt M, Lindeboom L, Phielix E, Havekes B, Wildberger JE, Hesselink MKC, Schrauwen P, Schrauwen-Hinderling VB. Carnitine supplementation improves metabolic flexibility and skeletal muscle acetylcarnitine formation. *In preparation*.
2. **Bruls YMH**, Op den Kamp YJM, Phielix E, Lindeboom L, Havekes B, Wildberger JE, Hesselink MKC, Schrauwen P, Schrauwen-Hinderling VB. L-carnitine infusion does not alleviate lipid-induced insulin resistance and metabolic flexibility. *In preparation*.
3. de Ligt M, **Bruls YMH**, Hansen J, Habets MF, Havekes B, Nascimento EBM, Moonen-Kornips E, Schaart G, Schrauwen-Hinderling VB, van Marken Lichtenbelt W, Schrauwen P. Resveratrol improves ex vivo mitochondrial function but does not affect insulin sensitivity or brown adipose tissue in first degree relatives of patients with type 2 diabetes. *Molecular metabolism*. 2018;S2212-8778(18):30221-7.
4. Lindeboom L, **Bruls YMH**, van Ewijk PA, Hesselink MK, Wildberger JE, Schrauwen P, Schrauwen-Hinderling VB. Longitudinal relaxation time editing for acetylcarnitine detection with (1) H-MRS. *Magnetic resonance in medicine*. 2017;77(2):505-10.
5. Brouwers B, Schrauwen-Hinderling VB, Jelenik T, Gemmink A, Havekes B, **Bruls YMH**, Dahlmans D, Roden M, Hesselink MKC, Schrauwen P. Metabolic disturbances of non-alcoholic fatty liver resemble the alterations typical for type 2 diabetes. *Clinical science*. 2017;131(15):1905-17.
6. Brouwers B, Schrauwen-Hinderling VB, Jelenik T, Gemmink A, Sparks LM, Havekes B, **Bruls YMH**, Dahlmans D, Roden M, Hesselink MKC, Schrauwen P. Exercise training reduces intrahepatic lipid content in people with and people without nonalcoholic fatty liver. *American journal of physiology Endocrinology and metabolism*. 2018;314(2):E165-e73.

ABOUT THE AUTHOR

Yvonne Bruls (MSc) was born in Heerlen, the Netherlands, on July 27th 1990. After graduating from the Sintermeerten College in Heerlen in 2008, she started her bachelor Health Sciences at the Maastricht University, with specialization in Movement Sciences and Biological Health Sciences. In 2011 she graduated and received the Top 3% award for belonging to the top 3% of best students of the Faculty of Health Medicine and Life Sciences.

Subsequently, she started the Master Physical Activity and Health with the specialization in both Biology of Human Performance and Health, and Sports and Physical Activity Interventions. During her master Internship she investigated exercise effects on intrahepatic lipid content and hepatic metabolism in relation to obesity, type 2 diabetes and non-alcoholic fatty liver disease. This internship resulted in publications in Clinical Science and the American Journal of Physiology, Endocrinology and Metabolism.

In September 2013, Yvonne was appointed as a PhD student at the department of Radiology and Nuclear Medicine at the Maastricht University Medical Center, the Netherlands. Yvonne worked, under supervision of dr. Vera Schrauwen-Hinderling, Prof. dr. Patrick Schrauwen, Prof dr. Matthijs Hesselink and Prof. dr. Joachim Wildberger, on her research project entitled: Acetylcarnitine: the role in metabolic flexibility and insulin sensitivity. Her work was in close collaboration with the department of Nutrition and Movement Sciences and conjoins clinical interventions with MR spectroscopy techniques to non-invasively investigate metabolic disturbances in humans.

Yvonne will continue her career as a postdoctoral researcher at the department of Radiology and Nuclear Medicine at the Maastricht University Medical Center. She will perform a human clinical intervention study with carnitine in patients with Type 2 Diabetes.