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Chapter 1

General introduction

Type 2 Diabetes Mellitus

A worldwide increase in people who are overweight or (morbid) obese remains a public health concern in today's society (1). As a result of an imbalance between intake of high-caloric meals and low physical activity, obesity drives an increase in several chronic diseases as cardiovascular disease, fatty liver disease and Type 2 Diabetes Mellitus (T2DM) (2, 3). In 2000, 171 million people worldwide were estimated diagnosed with T2DM, with an expectancy of 366 million people with T2DM in 2030 (4). However, in 2017 there were an estimated 451 million people with T2DM already (5), proving that the rate of increase is higher as expected. Within these past two decades, several new classes of antidiabetic drugs have been introduced, such as dipeptidylpeptidase-4 (DPPIV) inhibitors, glucagon-like peptide-1 (GLP-1) agonists, peroxisome proliferator-activated receptor (PPAR) agonists and sodium glucose transport protein 2 (SGLT2) inhibitors. Despite the availability of these new classes, the exponential growth of T2DM does not seem to attenuate (6). With most of these classes used as second or third-line therapy (7), and used to decrease HbA_{1C} levels as treatment goal (7), only patients with progressed T2DM are treated with these new antidiabetics. T2DM is characterized by a multiple-organ pathology, with defects in skeletal muscle, liver, pancreas, kidneys and gastrointestinal tract (7). With the availability of new antidiabetics, known to have their effect on different pathways and tissues (6), the question is if guidelines should aim at tackling specific tissue pathology, rather than only maintaining glycaemic control.

Ectopic fat accumulation and insulin resistance

Grasping back at the etiology of T2DM development, storage of excessive fat in patients with obesity is a major contributor. Especially the deposition of fat in peripheral tissues as for example skeletal muscle and liver, also known as ectopic fat accumulation, contributes to metabolic disturbances and development of insulin resistance (8, 9). Especially the development of insulin resistance in skeletal muscle, normally accounting for ~80% of the postprandial insulin-stimulated glucose uptake, is pivotal in the development of T2DM (9). This so-called lipid-induced insulin resistance is known to be multifactorial and the exact mechanism is still not entirely elucidated (10). However, it seems that the balance between lipid uptake into skeletal muscle and lipid oxidation inside skeletal muscle is impaired, resulting in a relatively low lipid-turnover in skeletal muscle. For example, patients with T2DM are known to have an impaired fat oxidative capacity in skeletal muscle (11), resulting in higher ectopic fat accumulation as a result

of incomplete β-oxidation. Subsequently, storage of lipid intermediates as diacylglycerol (DAG) and ceramides, are reported to impair insulin signalling, dependent of localization within skeletal muscle cells (12). Not only is there a necessity to understand the exact mechanisms behind lipid-induced insulin resistance in skeletal muscle. It is also important to find a suitable treatment to prevent the development of, or to attenuate the existence of, lipid-induced insulin resistance.

Treatment of lipid-induced insulin resistance

Several classes of medications are identified to act upon skeletal muscle lipid metabolism, either through higher lipid oxidation in skeletal muscle or through decreasing fatty acid availability towards skeletal muscle. A possible treatment option for improving lipid oxidation in skeletal muscle, is alleviating incomplete β-oxidation by carnitine treatment. Several rodent models, characterized by metabolic inflexibility and insulin resistance (13, 14), have assessed reduced free carnitine availability in skeletal muscle. Free carnitine availability is supposedly important to form acetylcarnitine and prevent intra-mitochondrial accumulation of acetyl-CoA, of which the latter could induce metabolic inflexibility (14, 15). Carnitine treatment has been shown to alleviated metabolic inflexibility and insulin resistance (13, 16), as well to improve incomplete β-oxidation in skeletal muscle (17), possibly caused by increasing both free carnitine levels and acetylcarnitine in skeletal muscle (18). Another class of drugs that could act upon lipid metabolism are the SGLT2 inhibitors. Whereas carnitine specifically acts upon lipid metabolism and affects lipid oxidation, SGLT2-inhibitors exert their effect on whole-body lipid metabolism. SGLT2-inhibitors increase urinary glucose excretion, and thus energy loss, thereby creating calorie-restriction like effects. Treatment with SGLT2-inhibitors are reported to increase glucagon levels (19), higher whole-body lipid oxidation (20), decreased liver fat content (21) and increased insulin sensitivity (20, 22). Since these results resemble calorie-restricted effects, the hypothesis here is that SGLT2-inhibition increases lipolysis and whole-body lipid oxidation, and subsequently increases lipid-turnover. As a result, it is expected that ectopic lipid accumulation decreases and insulin sensitivity improves.

Thesis outline

This thesis mainly focuses on the role of lipid-turnover in skeletal muscle tissue and its role in the development of lipid-induced insulin resistance. In **chapter 2** we reviewed the effect of several supplements and medication on lipid turnover and levels of lipid

intermediates. With this, modulation of lipid stores in skeletal muscle could provide a potential strategy to attenuate insulin resistance and improve T2DM treatment. In **chapter 3**, we focus on the role of specific lipid intermediates in skeletal muscle, and the accumulation of lipid intermediates in different subcellular locations. To this end we aimed to unravel the so-called athletes' paradox, where insulin sensitive endurance trained athletes have similar IMCL levels compared to insulin resistant sedentary participants, and investigated the difference in lipid intermediates and their subcellular localization between these populations. In **chapter 4** 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. In **chapter 5** we aimed to investigate the long-term effects of oral carnitine treatment in T2DM patients and describe the results of three months of carnitine supplementation on insulin sensitivity and metabolic flexibility and investigated if such effects are mediated by increased formation of acetylcarnitine. In **chapter 6** we investigated the physiological effects of a SGLT2-inhibitor on both whole-body and in skeletal muscle insulin sensitivity and substrate metabolism. With a double-blind, randomized, placebo-controlled cross-over trial, we investigated if SGLT2-inhibition is able to increase lipid oxidation and reduce (ectopic) lipid accumulation, thereby improving lipid-turnover and insulin sensitivity. Whether these whole-body physiological adaptations, as a result of energy loss, also result in skeletal muscle adaptations, is described in **chapter 7**. We investigated what the effects of SGLT2-inhibition are on skeletal muscle substrate utilization, oxidative capacity and lipid droplet localization.

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Chapter 2

Supplements and medication for improving lipid-turnover, alleviating IMCL and improving insulin sensitivity

Yvo J.M. Op den Kamp, Esther Phielix, Patrick Schrauwen

INTRODUCTION

In today's society, a western lifestyle including high-caloric meals combined with low physical activity, is the driving factor causing increased prevalence of several conditions, such as nonalcoholic fatty liver disease, atherosclerotic heart disease, obesity and T2DM (1, 2). In 2017, the prevalence of both diabetes and prediabetes globally reached 8.4% and 7.7% respectively (3), in which the development of insulin resistance plays a central role. The etiology of insulin resistance is known to be multifactorial, and is largely caused by metabolic impairments in several target tissues (4). Moreover, whole-body insulin resistance is known to be the most important early predictor of type 2 diabetes onset. Skeletal muscle normally accounts for ~80% of the postprandial insulin-stimulated glucose uptake, and therefore plays a predominant role in the development of insulin resistance (5). Lipid accumulation inside the muscle contributes majorly to metabolic disturbances and development of skeletal muscle insulin resistance (2). Human studies using MRS measurements, but also histochemical analysis in muscle biopsies have shown that intramyocellular lipid (IMCL) levels negatively correlate with insulin sensitivity (6, 7). The pathology of lipid-induced insulin resistance is complex and not merely a result of high levels of lipid content (7, 8). For example, levels of IMCL in endurance trained athletes are reported to be similar or even higher, compared to patients with T2DM or participants with insulin resistance (7). Both, lipid turnover and presence of specific lipid species, rather than IMCL content levels per se, underlie the development of lipid-induced insulin resistance. Modulation of lipid stores in skeletal muscle could potentially be a strategy to attenuate insulin resistance, aiming to improve T2DM treatment. In this review, we first elaborate on the development of lipid-induced insulin resistance as well as the role of lipid turnover and the specific lipid species involved. Second, we will evaluate the effect of several supplements and medication on modifying lipid turnover and lipid species in humans.

RELATION BETWEEN CELLULAR LIPID STORAGE AND INSULIN RESISTANCE

Lipid-induced insulin resistance seems not merely a result of higher FA uptake and aberrant deposition of fatty acids (FA) within skeletal muscle. A possible hypothesis to explain lipid-induced insulin resistance is a mismatch between skeletal muscle FA uptake and oxidative capacity, also termed lipid turnover, which could determine the level of IMCL deposition. Specifically, the mismatch between FA oxidation and the flux of tricarboxylic acid (TCA), could disrupt the balance in lipid turnover towards higher IMCL accumulation and could underlie the disturbances in lipid-induced insulin resistance.

Fat oxidative capacity and IMCL

In the last two decades or so, reduced fat oxidative capacity or specifically a diminished mitochondrial function has been linked to lower long-chain acyl-CoA shuttling towards mitochondrial β-oxidation, but rather towards synthesis of lipid intermediates (9). Indeed, studies have shown that participants with skeletal muscle insulin resistance, have lower mitochondrial activity (10, 11), structural mitochondrial damage and decreased content of mitochondria (12-15). However, whether low mitochondrial function precedes the development of insulin resistance has been challenged. It has been shown that blocking β-oxidation in skeletal muscle is capable of improving insulin sensitivity, despite increased IMCL accumulation (9, 16, 17), although this phenomenon can mainly be explained by substrate competition between fat and carbohydrate oxidation. Further evidence against a prime role of mitochondrial function in insulin resistance comes from studies in rodents chronically fed a high-fat diet, which resulted in increased capacity for mitochondrial fatty acid oxidation, accompanied with development of insulin resistance (9, 18, 19), suggesting that skeletal muscle aims to compensate for the increased availability of lipids. However, high fat diets do increase IMCL, suggesting that fat supply still outweighs oxidation (20). Under such conditions, fatty acid intermediates may accumulate and as already discussed, presence of specific lipid intermediates are known to affect insulin sensitivity. Indeed, the deposition of DAG species and ceramides are increased in patients with T2DM and in insulin resistant obese humans, when compared to lean participants (8).

Diacylglycerol and PKC

In the last decades, reports showed that the accumulation of diacylglycerol (DAG) plays a key role underlying lipid-induced insulin resistance. Moreover, specific DAG isoforms activate protein kinase C (PKC), subsequently followed by inhibition of insulin signaling, resulting in a diminished muscle glucose uptake (21). Especially translocation of PKC within the cell to the membrane upon activation seems to be related to insulin resistant skeletal muscle, as shown in human studies reporting a correlation between insulin sensitivity and translocation of PKC (22, 23). PKCθ and PKCϵ, the so-called novel PKCs, both require DAG for full activation and are believed to be the key species involved in the development of insulin resistance (8, 24). It has been suggested as well that stereoisomer type of DAGs and subcellular localization of DAGs within skeletal muscle affects insulin sensitivity. In line, an observational study including patients with T2DM, obese subjects, lean controls and athletes, reported increased sn-1,2-DAG levels in the sarcolemmal fraction of patients with T2DM (25). 1,2-DAG levels were higher in mitochondrial fraction of athletes, compared to obese subjects or T2DM patients (25). Translocation of PKC, upon activation by DAG species, is found to be involved in the inhibition of insulin signaling. More-over, PKCθ is reported to impair IRS1 tyrosine phosphorylation, through serine phosphorylation at several sites in skeletal muscle (26, 27). Evidence for the effect of specific DAG isoforms, cellular localization and subsequent activated PKCs on insulin sensitivity is limited and controversial.

Ceramides

Ceramides, also a byproduct of lipid intermediates after incomplete β-oxidation, are suggested to inhibit insulin signaling upon accumulation within skeletal muscle. A possibly site of action upon ceramide accumulation is inhibition of insulin receptor (INSR) tyrosine kinase activity (28, 29), or inhibition on the level of RAC-alpha serine/threonine-protein kinase (AKT) (30, 31), although evidence is scarce. Several species of ceramides are identified as related to insulin resistance. For example, C18:0 ceramides, which are especially located in the sarcolemmal, mitochondrial and nuclear compartments, have been found to correlate with insulin resistance (25, 32, 33). Also C18:1 and C16:0 ceramides are reported to correlate with insulin resistance (34). However, studies using myriocin as an inhibitor of ceramide biosynthesis upon palmitate infusion, only partially improved insulin resistance, suggesting that ceramide is not the only mediator in lipid-induced insulin resistance (35, 36).

What becomes apparent, is that IMCL and accumulation of DAGs and ceramides play a role in the development of insulin resistance. Whether IMCL or accumulation of lipid intermediates can be reduced, and if this subsequently leads to reduced lipid-induced insulin resistance, is understudied. In upcoming sections, several supplements and medication will be discussed, to elaborate on their potential effect to improve the above mentioned lipid-induced processes and with this, possibly improve insulin sensitivity.

NAD^+ precursors

Nicotinamide-adenine-dinucleotide (NAD^+) is an important substrate for sirtuin deacetylase activity, and with this, necessary for regulating metabolic homeostasis (37). In rodent models, supplementation with precursors of NAD^+ has been shown to enhance oxidative metabolism in skeletal muscle (37-39), reflected by enhanced mitochondrial biogenesis and increased oxidative gene expression. In myocardial tissue, impaired mitochondrial oxidative phosphorylation capacity has been associated with higher $NADH/NAD^+$ ratios (40), and intraperitoneal administration of nicotinamide mononucleotide (NMN) in mice with heart failure normalized NADH/NAD+ ratio and improved cardiac function (40). Another study showed that oral NR supplementation in a diet-induced obesity mouse model increased NAD^+ levels in adipose tissue and skeletal muscle, and simultaneously improved mitochondrial function (41). In animals, nicotinamide (NAM) and Nicotinamide Riboside (NR) supplementation, both precursors to NAD^+, have also shown to improve glucose metabolism, potentially providing an opportunity to boost mitochondrial function and lipid turnover in patients with obesity or T2DM (42).

So far, several intervention studies with NAD^+ analogues have been performed in humans. In a first study, it was found that 12 weeks of NR supplementation in obese and insulin resistant humans did not result in improved mitochondrial function (43). Similar results were found in a trial by Remie et al., where *ex vivo* mitochondrial function was unaltered with NR supplementation (44). Furthermore, Dollerup et al. (43) reported unchanged lipid deposition in skeletal muscle, both IMCL and EMCL, similarly to results reported by Remie et al. (44). In another trial of Dollerup et al. (45), NR administration did not lead to increased lipid oxidation and lipolysis rate, measured with a palmitate tracer, which matches to the finding of unaltered IMCL content (42, 43). Human trials show as well that NR supplementation does not change glucose rate

of disappearance (R_d) as a measure of insulin sensitivity, assessed with an euglycemic-hyperinsulinemic clamp (44, 43). On the contrary, in a placebo-controlled crossover trial in patients with T2DM, the administration of acipimox -another NAD^+ precursor and medication to treat hyperlipidemia- was able to increase mitochondrial oxidative capacity (46). Moreover, *ex vivo* mitochondrial state 3 and maximal uncoupled respiration increased upon acipimox treatment (46). Unexpectedly, skeletal muscle lipid content increased upon acipimox treatment, which could be attributed to increased fatty acid plasma levels, a well-known rebound effect of acipimox (46).

In conclusion, NAD^+ precursor supplementation in rodent models show positive results on mitochondrial respiration, but this effect has so far not been replicated in human studies. Subsequently, evidence from human trials do not support enhanced oxidative capacity, lipid-turnover or altered IMCL levels with NAD^+ precursor supplementation. NAD^+ precursor supplementation in rodent models show furthermore improved glucose metabolism and insulin sensitivity, however, this could not be reproduced in human trials.

Resveratrol

Similar to NAD^+ precursors, resveratrol is known to act upon the silent information regulator factor 2-related enzyme 1 (Sirt1) pathway (47, 48). Sirt1 induced deacetylase of peroxisome proliferator-activated receptor- γ coactivator-1α (PGC-1α) in skeletal muscle is able to regulate mitochondrial biogenesis and fatty acid utilization (49). Subsequently, oxidative capacity increases, which enhances lipid-supported mitochondrial respiration (50, 51). This hypothesis has been tested in a rodent study, where PGC-1α overexpression could potentially enhance insulin sensitivity through improved mitochondrial fatty acid oxidation (52). Rats fed a high fat diet intervened with a 8 week resveratrol supplement, had lower muscle lipid deposition in the tibialis anterior, specifically lower skeletal muscle free fatty acid and triglyceride content, compared to rats solely fed with a high fat diet (53). Besides lower lipid deposition, also enhanced subsarcolemmal mitochondrial β-oxidation, mitochondrial TCA cycle function and insulin sensitivity was observed in these rats (53). Together, these rodent studies indicate that resveratrol supplementation effectively improves both lipid and glucose metabolism in skeletal muscle (54).

Several human intervention studies with resveratrol supplementation have been reported. Timmers et al. (55) reported a trial with 11 healthy obese men, treated with 150 mg/daily of resveratrol for 30 days, and confirmed elevated SIRT1 and PGC-1α expression levels upon resveratrol administration, paralleled by enhanced activation of AMPK. In addition, Kjaer et al. (56) conducted a study with middle-aged men with metabolic syndrome, with either a placebo, low dose of 75mg resveratrol treatment twice daily or a high dose of 500mg resveratrol twice daily for 16 weeks. In the latter study PGC-1α expression levels were not determined, however, elevated SIRT levels could not be reproduced (56). Timmers et al. (55) reported furthermore increased skeletal muscle mitochondrial respiration *ex vivo*, only in the presence of a lipid substrate (55). Similar results were found in two other human trials, in which patients with T2DM (57), and first-degree relatives (58) underwent a 30-day treatment with 150mg resveratrol per day. More-over, in these populations mitochondrial respiration improved as well after adding octanoyl-carnitine as a substrate (57, 58). Regardless of resveratrol-induced expression of PGC-1α and improved mitochondrial respiration, IMCL content seemed unaffected or even elevated upon resveratrol treatment. Kjaer et al. (56) did not report changes in IMCL with resveratrol treatment, while others report increased IMCL levels in type 1 and type 2 muscle fibers (55, 57). Horsholm et al. (59) conducted a four-month placebo-controlled trial of resveratrol treatment, with a group treated with low dose of 75mg resveratrol twice daily and a group with high dose of 500mg resveratrol twice daily. Upon high dose of resveratrol, lower levels of intracellular lipids were found, however, only stearidonate and linolenate, which are both polyunsaturated FAs, were reported to be higher (59). Only one trial showed better insulin sensitivity measured with the HOMA-index upon resveratrol treatment, while three other trials reveal no changes in insulin sensitivity (56-58).

Where animal studies show improvement of insulin sensitivity or glucose metabolism after resveratrol treatment, possibly as a result of lower IMCL accumulation, a reduction in IMCL was not found in human intervention studies. In general, insulin sensitivity in human studies did not improve, regardless the dose of resveratrol supplementation. Nevertheless, repeatedly it has been shown that resveratrol improves mitochondrial respiration, in which both Sirt1 and AMPK seem to be essential players.

Poly unsaturated fatty acids

Muscle insulin sensitivity is well known to be associated with lifestyle factors, such as the type of diet. Dietary intake and the composition of nutrients in a meal are relevant and may increase IMCL content via enhanced supply of FAs into the muscle. Moreover, in rats, a diet enriched with saturated FAs, resulted in increased intramyocellular lipid accumulation, paralleled by elevated DAG and ceramides levels (60). However, aside from the diet-induced enhanced FA-supply into the muscle, the diet composition may affect hepatic lipid metabolism and hepatic TG output, thereby modulating IMCL. Indeed, diets enriched with fish oils, rich in long-chain ω-3 polyunsaturated FAs are known to alter hepatic triglyceride production and could potentially alter TG accumulation in skeletal muscle (61). The ω-3 polyunsaturated FAs are suggested to regulate FA oxidative capacity, via activation of PPARs (62) thereby regulating the expression of genes involved in FA metabolism. As a result, mRNA expression of PPAR regulated genes, such as mitochondrial transcription factor A, carnitine palmitoyltransferase-I (CPT-I) and fatty acid translocase (FAT/CD36) are enhanced (63-66). Furthermore, polyunsaturated FAs stimulate CPT-I activity, allowing higher fatty acid oxidation, lower malonyl-CoA inhibition and higher mitochondrial function (67, 68). These data support the notion that dietary PUFA's may modulate both oxidative capacity and lipid transport, at least in animals, thereby favoring lipid turnover through enhancement of oxidative capacity within skeletal muscle. A study with male Wistar rats found higher TG levels in the high-fat diet group with saturated FA compared to a high fat diet with saturated and short ω-3 FA (69). The effect of dietary PUFA's on IMCL seems however inconsistent, as some but not all studies show beneficial effects on lowering lipid accumulation within skeletal muscle (39, 70, 71), or lower DAG levels (72), whereas rodent studies with high fat feeding, rich in saturated FAs, report development of insulin resistance (73-76).

In humans, only a few studies addressed the effect of PUFA enriched diets on lipid turnover and insulin sensitivity. Interestingly, in one of these studies a dual-stable isotope tracer infusion approach was performed in insulin resistant men to assess both lipid uptake and fractional synthetic rate of lipids in skeletal muscle (77). Moreover, in this randomized crossover design participants received a liquid high-fat mixed meal enriched with either SFAs, MUFAs or PUFAs. No differences were reported for TAG, DAG, phospholipids or FFA levels, neither IMCL content differed between meals. The uptake of triacylglycerol-derived FA after a PUFA meal was lower than after a SFA meal.

Postprandial plasma glucose and insulin levels and insulin sensitivity were higher upon a SFA meal when compared to a PUFA enriched meal.

In rodent models, positive correlations were found between glucose metabolism, insulin sensitivity and lipoic acid supplementation. In line, in patients with T2DM, lipoic acid supplementation did lead to improvement in insulin sensitivity (78, 79). In addition, insulin sensitivity improved in patients with T2DM upon intravenous infusions of α lipoic acid (ALA) (80, 81). Altogether, more human trials are needed to assess the effect of PUFAs on lipid turnover and mitochondrial respiration. PUFA enriched diets seem to carry out an effect large enough to alter skeletal muscle lipid metabolism, although it needs to be determined whether this results in lower IMCL and/or lipid intermediates. Also, the effect on oxidative capacity needs more investigation. Perhaps oxidative capacity, and with this also lipid turnover, could be improved through a direct effect of poly-unsaturated FAs in skeletal muscle. Another explanation could be a decreased fatty acid level in plasma, originated from altered hepatic lipid metabolism. Nonetheless, PUFA enriched diets or infusion of (α) lipoic acid have a positive effect on insulin sensitivity, with strong clues of altered lipid turnover.

Carnitine metabolism

As previously described, a mismatch between FA oxidation and the flux through the TCA, results in an incomplete fatty acid oxidation and accumulation of lipid intermediates (9, 82). Another marker of incomplete fatty acid oxidation is the formation acylcarnitine (9). There is no clear evidence that increased levels of acylcarnitine are inducing insulin resistance directly. Carnitine plays an important role in the formation and transport of the acylcarnitines. After formation of acylcarnitines from (long-chain) FAs, acyl groups can be transported to either the mitochondrial matrix for β-oxidation, or towards plasma. It is known that lipid induced stress in skeletal muscle is able to inhibit CPT1 activity, and lower the rate of acylcarnitine formation (82, 83). The coupling of acyl-CoA to carnitine lowers the mitochondrial acetyl-CoA/CoA ratio which stimulates PDH activity thereby increasing glucose oxidation, and this releases mitochondrial oxidative stress (84). Carnitine supplementation is suggested to increase levels of free carnitine in skeletal muscle, which can be used for acylcarnitine formation. With this, potentially higher transport of acyl groups towards the mitochondrial matrix could result in more complete fatty acid oxidation resulting in less formation of lipid

intermediates. One rodent study shows improved and more complete lipid oxidation in skeletal muscle after carnitine supplementation (85). This is in line with another study, which reported that carnitine supplementation lowered TG levels in skeletal muscle, as well as FFA, resulting in improved insulin sensitivity (86). Several other animal studies, but not all (85, 87), that investigated the effects of carnitine supplementation showed improvement in insulin sensitivity and/or glucose tolerance (88-90).

Carnitine supplementation in human studies show a general positive effect on glucose tolerance or insulin sensitivity, especially in studies with T2DM patients (91-97). Bruls et. al. (98) conducted a study with impaired glucose tolerant (IGT) humans, with either placebo or 2g/day of L-carnitine supplementation in a crossover design, and added a group of normal glucose tolerant humans as a control group. It was found that plasma free carnitine and acetylcarnitine levels increased upon carnitine supplementation, as well as the acetylcarnitine formation in muscle tissue as measured using ^{1}H-MRS (98). Complete profiling of acylcarnitine species within skeletal muscle revealed a specific elevation of long-chain acylcarnitine levels in the IGT group compared to NGT subjects, indicative for incomplete β-oxidation(98). Interestingly, in the IGT group who received carnitine supplementation, a reduced content of long-chain acylcarnitines was measured when compared to placebo (98). In line, in the fasted state of the carnitine supplemented group, lipid oxidation appeared to be higher compared to placebo (98). Together, this point towards more complete fatty acid oxidation after carnitine supplementation. Also, metabolic flexibility was measured in the same participants, reflected by the insulin-stimulated change in RER versus baseline (98). The delta RER appeared to be lower in the group of IGT compared to NGT, which was restored upon carnitine supplementation (98). Therefore, results indicate that carnitine beneficially affects mitochondrial substrate switch resulting in improved whole body metabolic flexibility. However, carnitine supplementation did not result in elevated insulin-stimulated glucose uptake, as measured with a hyperinsulinemic-euglycemic clamp (98). Whether carnitine supplementation enables a reduction in levels of lipid intermediates needs to be determined.

Metformin

Metformin is the most used first-line therapy in treatment for T2DM patients, known to have effective glucose-lowering abilities and a good safety profile, without patients gaining weight as a result of treatment (99). The glucose-lowering abilities of metformin are likely caused by a variety of molecular mechanisms. One of the proposed mechanisms, is the direct and indirect activation of AMPK in liver, via inhibition of complex I in mitochondria (100). In the liver, AMPK is suggested to stimulate lipid oxidation and to inhibit both lipid synthesis (101) and gluconeogenesis (102). Less is known about the metformin effects on AMPK in skeletal muscle. Since metformin is suggested to reduce lipid deposition in liver, it is tempting to suggest that metformin also has an effect on improvement of skeletal muscle lipid deposition and subsequently improvement on glucose homeostasis (103, 104). Indeed, in hepatocytes metformin is known to activate AMPK and a similar effect could be anticipated in skeletal muscle (105, 106). AMPK activation is also able to increase PGC-1α protein content and increase mitochondrial biogenesis, resulting in improved lipid oxidation (107, 108). Rodent studies show metformin-induced AMPK activation in skeletal muscle (102, 109) and decreased levels of skeletal muscle DAG and ceramide levels (104-106), lower PKCθ translocation towards muscle cell membrane (104), and lower accumulation of acyl-CoA in skeletal muscle (106). These metformin-induced improvements in lipid-turnover were furthermore paralleled by improved insulin sensitivity or insulin tolerance (104, 106). Therefore, beside the plasma glucose lowering effects in patients with T2DM, metformin also potentially leads to improved lipid-turnover, thereby enhancing insulin sensitivity.

Only a few studies performed in T2DM patients focused at metformin-induced changes in lipid accumulation. Teranishi et. al. (110) performed a study with 6 months of metformin treatment and reported lower IMCL values after treatment, measured using ^{1}H-MRS. However, in another trial of Tamura et al. (111), levels of IMCL in tibialis anterior muscle did not change after metformin treatment. Differences in severity of glucose intolerance characterizing the patients with T2DM could underlie the difference in IMCL levels between the two studies, since Teranishi et. al. (110) included patient with more severe T2DM and two-fold higher basal IMCL values compared to the study of Tamura et. al. (111). Both trials reported an improvement of insulin sensitivity or glucose infusion rate during an euglycemic hyperinsulinemic clamp (110, 111). The question remains whether the increased insulin sensitivity upon metformin treatment is a result of improved lipid-turnover in skeletal muscle or could be the resultant of reduced

glucotoxicity through glucose lowering effects.

PPARα agonists

Fibrates are widely used as a treatment for dyslipidemia. Fibrates are agonists of the peroxisome proliferator-activated receptor (PPAR) α (112), which is essential for the transcription of genes implicated in lipid and lipoprotein metabolism in the liver (113). PPARα activation increases lipoprotein lipase (LPL) activity in the liver, enhancing FA catabolism and limiting TG synthesis, which affects VLDL production as well (114). Also, FA uptake and LDL removal by the liver is upregulated upon PPARα activation (114, 115). Altered lipid and lipoprotein metabolism in liver therefore has a great effect on plasma TG and FA levels (116). Since PPARα is expressed in a wide range of tissues and regulates expression of several genes involved in lipid metabolism, an increase in lipid oxidation is also expected in skeletal muscle (112, 117). Whether PPARα influences lipid metabolism in skeletal muscle and affects insulin sensitivity, either directly via activation of PPARα in skeletal muscle or indirectly through lower plasma FA levels, is unclear. Rodent studies have shown that fenofibrate, a well-known PPARα agonist, lowers lipid content within skeletal muscle (112, 118-120). More specifically, upon fenofibrate treatment, malonyl-CoA levels reduced ~27% in skeletal muscle cells of pre-diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats (121). Also other PPARα agonists, like bezafibrate and pirinixic acid lower IMCL levels upon treatment (122, 123). Furthermore, based on these studies, PPARα agonists seem to improve glucose uptake (112), insulin sensitivity (120-123), whole-body glucose tolerance (118), accompanied by significant lower blood glucose and insulin levels (119).

Although several rodent studies with PPARα agonists have been performed, studies investigating the effect on skeletal muscle lipid accumulation and insulin sensitivity in humans are scarce. Perreault et al. (124) performed a study with fenofibrate treatment in IGT participants and measured IMTG levels, lipid composition and synthesis rates of IMTG (124). Results pointed towards a gender-specific fenobribrate effect, with no change in IMTG levels and increased fractional synthetic rate (FSR) (of IMTG) in male participants (124). Despite the marginal influence on IMTG levels, insulin sensitivity did improve upon fenofibrate treatment (124). The question remains if lowering of TG content in skeletal muscle is predominantly caused by a treatment effect on the liver or skeletal muscle, especially since expression of PPARα is mostly seen in liver, heart and

kidney, and less in skeletal muscle (125).

PPARγ agonists

Thiazolidinediones (TZD) are potent antidiabetic drugs with affinity for the PPARγ receptor. From animal studies with T2DM models it is known that TZDs attenuate hyperglycemia, hyperinsulinemia and hypertriglyceridemia (126). It is proposed that PPARγ agonist exert their main action via adipose tissue, through several pathways. PPARγ activation is able to induce differentiation of preadipocytes into adipocytes (127). Also, activation of PPARγ stimulates storage of FAs in adipose tissue, as a result of higher plasma triglyceride clearance mediated by enhanced LPL activity (128). PPARγ-stimulated fatty acid uptake in adipose tissue could hypothetically result in a lower FA flux towards muscle, resulting in lower IMCL. Studies have shown that TZD treatment increases adiponectin secretion from mature adipocytes (129), a hormone known to improve insulin sensitivity and glucose utilization, probably through improved lipid turnover by increasing skeletal muscle fatty acid oxidation (130, 131). With this, PPARγ agonist treatment is suggested to improve lipid-turnover through enhancement of oxidative capacity and lower FA uptake into skeletal muscle. In line, rodent studies have reported lower TG (123, 132, 133) and ceramide content in skeletal muscle (134) upon pioglitazone treatment. Treatment with rosiglitazone did not alter TG content in skeletal muscle (135-137), however, lower levels of DAG and saturation of FA and DAG were found (136). Treatment with these compounds in rodents revealed improved insulin sensitivity (132, 135).

Pioglitazone treatment in patients with T2DM resulted in lower IMCL and long-chain acyl-CoA levels, without changes in DAG (110, 138). Similar to results in rodent studies, rosiglitazone treatment did not alter IMCL levels in vastus lateralis muscle of T2DM patients or IGT participants (139-142). However, it seems that relative levels of saturated long-chain FAs were lower after rosiglitazone treatment, with higher relative levels of unsaturated long-chain FAs (142). Also, higher rates of lipid oxidation have been reported in the fasted state, together with lower accumulation of malonyl-CoA upon PPARγ treatment (143). Eventually, both rosiglitazone and pioglitazone are able to improve skeletal muscle insulin sensitivity or increase insulin-stimulated glucose uptake after treatment (110, 138-141). Whether PPARγ agonists beneficially affect insulin sensitivity through adiponectine production or is merely a consequence of

enhanced lipid-turnover, is unknown.

PPARδ agonists

PPARδ is found in almost all high metabolic tissue and unlike PPARγ, also present in skeletal muscle (144). There are reports that PPARδ activation increases β-oxidation, enhances mitochondrial biogenesis and muscle fiber-type switching (144-147), but the working mechanism of PPARδ activation leading to improved insulin sensitivity is relatively unkown. PPARδ activation in skeletal muscle may alter the expression of multiple genes involved in lipid metabolism, such as fatty acid transport protein 1 (FATP1), long-chain acyl-CoA dehydrogenase, CPTI, and long chain acyl-CoA synthetase (62).

A rodent study using PPARδ activation showed higher lipid oxidation upon treatment (148) paralleled by higher mRNA expression of FATP, long-chain acyl-CoA dehydrogenase (LCAD), uncoupling protein 2 UCP2, uncoupling protein 3 UCP3 and peroxisome proliferator-activated receptor gamma co-activator 1 α (PGC-1α) (62). Simultaneously, lipid droplets around the mitochondria dissapeared almost completely (62). PPARδ treatment increases insulin sensitivity in rodent studies (62, 148, 149). On the contrary, one study reported lower insulin sensitivity after PPARδ activation (150).

Overall, evidence in animal studies point towards a PPARδ-stimulated lipid oxidation rate, increased mitochondrial biogenesis and reduced lipid accumulation or lowered accumulation of lipid intermediates. Use of PPARδ agonists in human trials would definitely be interesting, since expression of PPARδ in skeletal muscle is relatively high, compared to other PPARs. However, side-effects of PPARδ in humans so far has prevented the use of these agonist in human clinical experimental trials.

Acipimox

Acipimox is a nicotinic acid analog used to treat patients with hyperlipidemia. Acipimox acts as an inhibitor of lipolysis (151), concomitantly reducing plasma FA levels. However, a major issue with acipimox treatment is the well-described rebound effect, meaning that after a prolonged time of treatment, circulating fatty acid levels are in fact increased instead of decreased. Similarly to the effect of PPARγ agonists, it is

hypothesized that lower FA levels in plasma result in lower FA uptake in skeletal muscle. Walker et al., (152) tested this hypothesis with use of a palmitate tracer and indeed found reduced FA uptake in muscle upon acipimox treatment. Treatment of acipimox is hypothesized to attenuate insulin resistance, by lowering plasma FA levels. This is not only found in rodent models (153, 154), but also in numeral human studies (155-157), even if treatment was short-term (158).

Bajaj et al. (159) reported a sustained reduction of plasma FA levels with 7 days of acipimox treatment in T2DM patients. Skeletal muscle long-chain FA-CoA's were reported to be lower after treatment (159). Surprisingly, specific analysis of these LCFA-CoA revealed that not only saturated, but also unsaturated FA's were decreased after treatment. Paradoxically, the only FA that was not significantly altered, was stearate CoA (18:0) (159). In addition, whole body lipid oxidation was decreased in the basal state, which matches the reduced lipolytic effect of acipimox. Acipimox treatment seemed to be able to improve insulin sensitivity, mainly through increased nonoxidative glucose disposal (159). Another study with similar treatment duration in insulin resistant subject with a family history of T2DM, obtained comparable results including lower plasma FA levels and skeletal muscle long-chain FA-CoA's paralleled by enhanced insulin sensitivity and nonoxidative glucose disposal (151). Above mentioned studies did not describe any NAD^+ effects, as a result of acipimox treatment. There is however, a clear difference in mitochondrial function between acute and chronic treatment with acipimox. ATP synthesis increased upon 12 days of acipimox treatment (155), while acute acipimox treatment did not markedly change *ex vivo* mitochondrial respiration (156). Possibly, chronic acipimox treatment is able to improve mitochondrial function, which is dissociated from improvement in insulin sensitivity. However, because of the well-described rebound effect, acipimox is not a realistic option for the long-term treatment of type 2 diabetes.

Statins

Statins are used as a first-line treatment for hypercholesterolemia in patients with high risk of cardiovascular disease (160). As an inhibitor of 3-hydroxy-3methyl-glytaryl-coenzyme A (HMG-CoA) reductase, biosynthesis of cholesterol in the liver is decreased. This results in lower cholesterol production and upregulation of LDL receptors and clearance of LDL-cholesterol out of the circulation (161). Besides the cholesterol

lowering effects, lower FA plasma levels are reported as well (162). Like other plasma FA lowering medication, it could be hypothesized that insulin sensitivity can be improved by lowering skeletal muscle FA uptake. However, the general positive effects of statins on plasma lipid composition do not lead towards improved metabolic health in skeletal muscle. It was found that the prevalence of T2DM in people on statin treatment is higher in those using lipophilic statins (i.e. simvastatin, atorvastatin, fluvastatin, lovastatin) (163-165), especially compared to those patients using hydrophilic statins (pravastatin, rosuvastatin) (166), eventhough not all studies that investigated the use of lipophilic statin report decreased insulin sensitivity (167). It is widely known that statines can have adverse effects on skeletal muscle, for example myositis, rhabdomyolisis and muscle pain (168). The mechanism behind the myopathic symptoms are not completely understood, but could involve higher ROS production and/or mitochondrial toxicity / dysfunction in skeletal muscle (169). Also, reduction in citrate synthase and mitochondrial content, together with lower coenzyme Q10 levels in skeletal muscle are reported after simvastatine treatment (170). These effects may contribute to the lack of beneficial effects of statins on muscle insulin sensitivity, despite the lowering of plasma FFA levels.

A rodent study with a lipophilic statin, fluvastatin, revealed higher IMCL accumulation upon treatment in streptozotocin induced diabetic mice, with down regulation of FAT/CD36 protein expression in skeletal muscle (171). In humans, case reports with both simvastatin and atorvastatin treatment and side effects of muscle pain, showed clearly increased lipid droplets, which resoluted after discontinuation of statin treatment (172). Another human study reported decreased CD36 and decreased DGAT1 and a tendency to decreased DGAT2 protein expression after statin treatment, accompanied with decreased insulin sensitivity, compared to control participants (173). Decreased levels of CD36 have been linked to lower coenzyme Q10 uptake, at least in brown adipose tissue (174). The only human study looking into the effect of statins on both insulin sensitivity and lipid accumulation in skeletal muscle of T2DM did not reveal any differences in both insulin sensitivity or IMCL upon treatment with simvastatin, despite lower FA plasma levels (175).

It is unknown whether hydrophilic statins are metabolically more beneficial than lipophilic statins in general, or whether impaired insulin sensitivity is only seen in patients with muscle symptoms. However, it has been shown that statins do not

improve metabolic health in skeletal muscle. Future human studies comparing both lipophilic and hydrophilic statins are warrented to investigate a possible effect on mitochondrial toxicity, lipid-turnover and insulin sensitivity.

GLP-1 agonists and DPP-4 inhibitors

GLP-1, a peptide produced by intestinal endocrine L-cells, is a glucose-dependent insulinotropic hormone, with co-occurrent effects of delayed gastric emptying and increased sense of satiety (176-178). The insulinotropic effect of GLP-1 is reduced in T2DM patients, caused by chronic hyperglycemia and hyperlipidemia-induced β-cell dysfunction (179-181). GLP-1 agonist treatment is able to improve glucose metabolism in T2DM through increased insulin secretion, but also other effects such as reduced body weight, decreased liver fat accumulation, and lowering of plasma cholesterol and FA levels are reported (182, 183).

Rodent studies studying the effect of GLP-1 agonist treatment show distinctive results on TG levels in skeletal muscle, with either no changes in TG content (181), or a dose-dependent decrease of TG content in skeletal muscle with a non-peptidic GLP-1 agonist (184). The result of GLP-1 agonist treatment on skeletal muscle insulin sensitivity differs between studies as well, with one study reporting no change in insulin sensitivity (181), and the other study reporting dose-dependent improvement of insulin sensitivity (184). The difference could possibly be explained by different treatment duration between the studies, with respectively two and twelve weeks. Another rodent study, also with exenatide treatment, reported ameliorated IMCL deposition and improvement of glucose and insulin tolerance after 4 weeks of treatment (185). Treatment of exenatide was tested in both ob/ob mice and C57BL/6 mice on an HFD, and the effect on lowering IMCL content was largest in mice fed an HFD. Analysis of skeletal muscle protein expression revealed marked increases in phosphorylated AMPK and ACC, accompanied with increased CPT1 expression and decreased expression of the lipogenic genes SREBP1c and FAS.

These results suggest that long-term use of GLP-1 agonists could potentially reduce lipid accumulation, at least in rodent models. It is still relatively unknown what the mechanism of reduced lipid accumulation is. Although use of GLP-1 agonist treatment results in lower plasma FA levels, which could result in lower FA uptake into skeletal

muscle, the increase in phosphorylated AMPK and ACC suggests higher oxidative capacity. With delayed gastric emptying and reduced satiety as an effect of GLP-1 agonists, decreased body weight has been reported as well (186). The reduction in body weight, could partially explain the effects on IMCL. The study of Xu et al. (187), reported a reduction in IMCL, without any changes in body weight upon GLP-1 treatment. Since GLP-1 is inactivated by dipeptidylpeptidase-4 (DPP-4), DPP4 inhibitors are another potential treatment option for patients with T2DM. However, currently, no studies on the effect of DPP4 inhibitors on lipid-turnover or insulin sensitivity in skeletal muscle are available.

DISCUSSION

Enhanced lipid-turnover via modulating skeletal muscle lipid stores potentially forms a strategy to attenuate insulin resistance. Lipid-induced insulin resistance caused by a mismatch between skeletal muscle FA uptake and oxidative capacity, is a generally well accepted hypothesis. Therefore, lowering skeletal muscle FA availability or increasing oxidative capacity may potentially modulate lipid stores in muscle beneficially affecting insulin sensitivity.

NAD precursors, resveratrol, carnitine and PPARδ agonists are compounds that have been described to exert an effect on skeletal muscle mitochondrial biogenesis and respiration, and could thereby affect IMCL levels. However, controversial effects are found upon treatment with either of these compounds, with rarely any effect on total IMCL or effect on lipid intermediates. Furthermore, insulin sensitivity did not substantially improve in either of these compounds, although carnitine supplementation showed mild improved metabolic flexibility and insulin sensitivity. Metformin also is suggested to activate mitochondrial respiration and metformin treatment outcomes show that direct stimulation of lipid oxidation or mitochondrial respiration seems to be insufficient to overcome lipid-induced insulin resistance in patients with T2DM.

PUFAs and PPARα agonists enhance lipid oxidation in skeletal muscle, but also affect TG and FA metabolism in the liver resulting in lower FA plasma levels. Therefore, improved insulin sensitivity could be exerted by improved lipid-turnover and or through lower FA availability, although the magnitude of contribution of each pathway is unknown. GLP-1 agonists and DPP4-inhibitors have the potency to create similar

effects, but more research is needed.
A reduction in FA availability towards skeletal muscle seems to be most effective in lowering IMCL resulting in improved insulin sensitivity. Both PPARγ agonists, and short-term acipimox treatment, prove to decrease FA plasma levels and subsequently decrease skeletal muscle FA uptake as their main effect. Especially these compounds show reduced IMCL stores, and lower amounts of total long chain FAs in skeletal muscle upon treatment. Higher amounts of long chain FAs are suggested to correlate with insulin resistance, since effective lowering of long chain FAs in skeletal muscle increased peripheral insulin sensitivity (159). So far, it is uncertain if reduction in IMCL or long-chain FAs lead towards alterations in DAG or ceramide levels. Most compounds described in this review, which increase insulin sensitivity show inconclusive results regarding DAGs and ceramides.

In conclusion, mainly PPARγ agonists and acipimox are able to improve insulin sensitivity, through modulation of lipid-turnover. Modulation of lipid-turnover by these types of medication is achieved by lowering FA availability to skeletal muscle.

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Chapter 3

Intracellular localization of lipid stores in human skeletal muscle of trained and untrained individuals

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Chapter 4

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

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ABSTRACT

Background: Low carnitine status may underlie the development of insulin resistance and metabolic inflexibility. Intravenous lipid infusion elevates plasma free fatty acid (FFA) concentration and is a model for simulating insulin resistance and metabolic inflexibility in healthy, insulin sensitive volunteers. Here, we hypothesized that co-infusion of L-carnitine may alleviate lipid-induced insulin resistance and metabolic inflexibility.

Methods: In a randomized crossover trial, eight young healthy volunteers underwent hyperinsulinemic-euglycemic clamps (40mU/m^2/min) with simultaneous infusion of saline (CON), Intralipid (20%, 90mL/h) (LIPID), or Intralipid (20%, 90mL/h) combined with L-carnitine infusion (28mg/kg) (LIPID+CAR). Ten volunteers were randomized for the intervention arms (CON, LIPID and LIPID+CAR), but two dropped-out during the study. Therefore, eight volunteers participated in all three intervention arms and were included for analysis.

Results: L-carnitine infusion elevated plasma free carnitine availability and resulted in a more pronounced increase in plasma acetylcarnitine, short-, medium-, and long-chain acylcarnitines compared to lipid infusion, however no differences in skeletal muscle free carnitine or acetylcarnitine were found. Peripheral insulin sensitivity and metabolic flexibility were blunted upon lipid infusion compared to CON but L-carnitine infusion did not alleviate this.

Conclusion: Acute L-carnitine infusion could not alleviated lipid-induced insulin resistance and metabolic inflexibility and did not alter skeletal muscle carnitine availability. Possibly, lipid-induced insulin resistance may also have affected carnitine uptake and may have blunted the insulin-induced carnitine storage in muscle. Future studies are needed to investigate this.

Keywords Insulin sensitivity, metabolic flexibility, L-carnitine infusion, hyperinsulinemic-euglycemic clamp, lipid infusion.

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). The infusion of lipids in insulin sensitive subjects temporarily causes lipid-induced insulin resistance and metabolic inflexibility, which is therefore a well-appreciated model to investigate the mechanisms underlying the development of insulin resistance and metabolic inflexibility (3, 4). It is well known that exercise-trained individuals are more insulin sensitivity and metabolically flexible compared to untrained BMI- and age-matched individuals (4, 5). Interestingly, when using a lipid infusion model, the degree of lipid induced insulin resistance and metabolic inflexibility differs between exercise-trained and untrained individuals. Exercise-trained athletes remained more insulin sensitive, reflected by a reduction in insulin sensitivity of 29% in compared to 63% in untrained individuals [4]. Why exercise-trained individuals remain more insulin sensitive and metabolic flexible upon lipid infusion is mechanistically still unclear. Possible, the availability of skeletal muscle free carnitine might play a role in here.

Recently, carnitine has been suggested to play a role in maintaining insulin sensitivity and metabolic flexibility (6-8). Although carnitine is best known for its function in the transport of long-chain fatty acyl-units into the mitochondrial matrix, allowing subsequent β-oxidation (9, 10), it exerts other functions as well. The function of carnitine to conjugate with acetyl-CoA to form acetylcarnitine is gaining increasingly interest as it may be relevant in preserving insulin sensitivity and metabolic flexibility (6). This conjugation to acetylcarnitine is facilitated by the enzyme carnitine acyl transferase (CrAT) in the mitochondria. With reduced acetylcarnitine formation, acetyl-CoA may accumulate inside mitochondria, especially during conditions of high substrate load (i.e. exercise or high-fat feeding). A rise in acetyl-CoA could inhibit the activity of pyruvate dehydrogenase (PDH), which is a rate-limiting step in the conversion of pyruvate into acetyl-CoA. As a consequence, mitochondria may be less able to maintain high rates of glucose oxidation, which may infer metabolic inflexibility. The availability of carnitine is therefore crucial in acetylcarnitine formation and may be actively involved in maintaining metabolic flexibility, insulin sensitivity and glucose homeostasis (6-8).

Animal studies indeed explored the link between metabolic inflexibility and changes in carnitine status but results are inconclusive (6, 7, 11-13). While some studies did not reveal a link between metabolic flexibility and changes in carnitine status (12, 13), Noland et al. showed that a reduction in free carnitine availability in rats was associated with decreased metabolic flexibility and that the consumption of a high-fat diet even could lower free carnitine availability (7). Interestingly, increasing carnitine availability via supplementation in these rats resulted in complete restoration of metabolic flexibility, as well as remaining high PDH activity and consequently insulin sensitivity (7). Furthermore, observational data showed that obese mice are characterized with low acetylcarnitine concentrations, which could underlie the insulin resistant state (6, 11). Interestingly, these animals benefit from improved carnitine availability as acetylcarnitine levels restored concomitantly with improved metabolic flexibility, PDH activity, insulin sensitivity, as well as blood glucose levels (11). The latter suggest better glucose homeostasis and therefore may be a pivotal mechanism to further explore in type 2 diabetes. In patients with type 2 diabetes, lower skeletal muscle acetylcarnitine concentrations were detected using magnetic resonance spectroscopy (1), which possibly indicate low carnitine availability. Carnitine supplementation has been shown to improve glucose tolerance in insulin resistant subjects with low carnitine status (15). Some, although not all studies, reported beneficial effects of carnitine administration on plasma glucose, insulin and lactate levels (16-20). Furthermore, markers of insulin resistance such as glucose infusion rate (GIR) (19, 20) and M-value (21) 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 L-carnitine infusion during simultaneous lipid infusion could alleviate lipid-induced insulin resistance and metabolic inflexibility in healthy young males.

METHODS

Ethical approval

Study procedures were approved by the Medical Ethical Committee in accordance with the declaration of Helsinki. The full name on the ethics committee is the medical-ethical review committee of the academic hospital Maastricht (azM) and Maastricht University (UM), METC azM/UM. Trial monitoring was performed by the Clinical Trial Center Maastricht. The study was registered at clinicaltrials.gov with identifier NCT02722902, full date of first registration was March 30, 2016. All subjects gave written informed consent after the protocol was fully explained.

General characteristics

Eight young (18-40 years), healthy sedentary lean (BMI: 18-25 kg/m^2) males participated in this study. Participants were excluded in case of medication use interfering with glucose homeostasis and/or study procedures, exercise engagement exceeding 3 hours a week, smoking, unstable body weight (weight gain or loss > 5kg in the previous 3 months), alcohol and/or drug abuse, impairments in kidney and/or liver function, uncontrolled hypertension, cardiovascular disease. Furthermore, significant food allergies/intolerances to the applied intervention, participation in another biomedical study within 1 month before the first study visit, and anaemia (haemoglobin levels <7.8 mmol/L) were exclusion criteria. Participants who intended to donate blood during the study period or participants who have donated blood less than three months before the start of the study were not included to minimize the risk of anaemia due to repetitive blood sampling in this study. Participants were not included in case they did not wanted their treating physician to be informed about participation in the study. Furthermore, if participants did not want to be informed about unexpected medical findings participation in the study was not possible. Finally, vegetarians were not included because of the altered whole body carnitine status.

Experimental design

The study was set up as a single blind, placebo-controlled randomized cross-over design. The study was conducted at Maastricht University Medical Center, The Netherlands, between December 2016 and June 2017. 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 came in the morning after an overnight fast. Body composition (fat percentage and fat free mass) was determined. Subsequently, maximal

oxygen uptake (VO_{2max}) and maximal power output were determined during an incremental cycling test on a stationary bike to determine training status. On each of the following visits (visit 2, 3 and 4), participants came in at 06:00 h after an overnight fast. On each of these visits (visit 2, 3 and 4), 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 study arms, 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. Block randomization with blocks consisting of three items (1: CON, 2: LIPID, 3: LIPID+CAR) was performed by the researchers as previously described by Snedecor and Cochran (22). Participants were blinded for treatment. The half-life of release of carnitine from skeletal muscle is 139 hours, therefore a wash-out period of at least two weeks was used to prevent carry over effects of the L-carnitine and lipid infusion. Primary outcome was the effect of additional L-carnitine in combination with lipid infusion on insulin sensitivity and metabolic flexibility compared to only lipid infusion. Secondary outcome measures were plasma and skeletal muscle acylcarnitine profiles.

Body composition

During the first visit, participants came in after an overnight fast. Body mass and body volume were assessed using air-displacement plethysmography (ADP) using the Bod Pod (Cosmed, Rome, Italy) according to the manufacturer's instructions and as described previously (23, 24). Thoracic gas volume was predicted based on equations included in the software. From these data, body composition (fat mass, fat free mass and fat percentage) was calculated as described by Siri (25).

VO_{2max}

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 (VO_{2max}) and maximal power output (W_{max}) as reported previously (26) for characterization of the participants and to confirm that the participants were not exercise-trained. 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 (Omnical, Maastricht, The Netherlands).

Hyperinsulinemic-Euglycemic clamp

At visit 2, 3 and 4, 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 standardized 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 06:00 h after an overnight fast from 20:00 h onwards. A fasted blood sample was obtained and a primed constant 6-hour insulin infusion was started (40mU/m²) with simultaneous infusion of variable amounts of glucose (glucose 20%) to maintain euglycemia (5.0mmol/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, Melsungen, Germany) was administrated at an infusion rate of 90ml/h. Intralipid consisted of pure soya-oil including linoleic acid, linolenic acid, oleic acid, palmitic acid and stearic acid. All included lipids are long chain triglycerides (LCT). A primed (4mg/kg) continuous (4mg/kg/h) infusion of L-Carnitine (Carnitene, Alfasigma, Utrecht, The Netherlands) 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 min) and the last 30 minutes (t=330-360 min) of insulin stimulation to determine metabolic flexibility and glucose and lipid oxidation rates according to Peronnet et al. (27). The respiratory exchange ratio (RER), defined as VCO_2/VO_2, was used to determine metabolic flexibility (ΔRER). Metabolic flexibility (ΔRER) reflects the difference between the insulin stimulated RER (t=330-360 min) minus RER at basal conditions (t=-30-0 min).

Muscle biopsies

On the day of the hyperinsulinemic-euglycemic clamp (visit 2, 3 and 4), skeletal muscle biopsies were taken upon 6 hours of insulin stimulation. In the control arm, an additional muscle biopsy was taken in the morning after an overnight fast. Muscle biopsies were taken from the m. vastus lateralis muscle according to the Bergstrom method (28) 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 (29). Total short-chain acylcarnitine species included the sum of C3 until C5 carnitine species. C6 until C12 acylcarnitine species were defined as medium-chain acylcarnitine species. Long-chain acylcarnitine species represented C14 until C18-acylcarnitine species.

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) concentrations were determined at t=120, 180, 240, 360 and 480 minutes via an enzymatic assay automated on a Cobas Dara/Mira analyzer (Wako Nefa C test kit, Wako Chemicals, Neuss, Germany). Plasma acylcarnitine species were measured using tandem mass spectrometry as previously described (30) during the basal and insulin stimulated steady state (t=120 and t=480 minutes) and at t=180 minutes to determine the expected increase in plasma acetylcarnitine levels. Total short-chain acylcarnitine species included the sum of C3 until C5 carnitine species. C6 until C12 acylcarnitine species were defined as medium-chain acylcarnitine species. Long-chain acylcarnitine species represented C14 until C18-acylcarnitine species.

Sample size calculation and study status

The sample size was calculated based on the results from previous carnitine infusion studies reporting clinically significant improvements in insulin sensitivity after carnitine infusion of 0.5-1.4 mg/kg/min during a hyperinsulinemic-euglycemic clamp (19, 20). The intraindividual variation (SD) of the difference in insulin sensitivity in repeated measurements is reported to be 0.68 mg/kg/min (31-33). To reach 80% power, assuming an improvement of 0.5 mg/kg/min, and a significance level of 0.05, a minimal calculated sample size of N=13 was needed. An interim-analysis was performed after eight participants completed the entire study with all three intervention arms (17 participants were recruited for screening by then), revealing no effect of the carnitine treatment. Therefore, the study was terminated prematurely after eight participants.

Statistics

The statistical analysis was performed using SPSS 24.0 software (SPSS, Chicago, Il.). All results are presented as mean ± 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 (ΔRER) and skeletal muscle acylcarnitine species between study arms. For the comparisons of skeletal muscle acylcarnitine species in the insulin-stimulated states with the basal state, a Student's paired sample t-test was performed with Bonferroni correction for multiple testing and therefore, p-values 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 analysis were performed.

RESULTS

Participant characteristics

Eight healthy young lean male participants (body weight = 76.5±1.9 kg, BMI = 23.2±0.4 kg/m^2, age = 22±1 year) were included. No drop-outs were reported. All participants had a sedentary life style (not engaged in regular physical activity). Their maximal oxygen uptake (VO_2max) (42.4±2.5 ml/min/kg) and body fat percentage (17.1±1.9%) were within the normal range for young, untrained males. Participant enrollment and allocation are presented in Fig 1 whereas characteristics are presented in table 1. No adverse events of the Intralipid and/or carnitine infusion have occurred in this study.

Insulin sensitivity

At baseline, plasma FFA levels were comparable between study arms (389±47 vs. 415±40 vs. 382±60 µmol/L in CON, LIPID and LIPID+CAR respectively, P=0.885). In the lipid trial an increase in FFA levels occurred over time and were significantly higher at all time points compared to the control condition (P <0.01, Fig 2A). Simultaneous infusion of L-carnitine did not alter FFA levels when compared to lipid infusion alone (P=0.939, Fig 2A). During the first three hours of the clamp, glucose infusion rates (GIR) were comparable between all three study arms (P=0.448 Fig 2B). From 3 hours onwards, glucose infusion rates were lower in LIPID as well as in LIPID+CAR compared to CON (P<0.01, Fig 2B), consistent with previous studies showing that lipid-induced insulin resistance occurs after 2-3 hours of lipid infusion (4, 34). No difference was found in GIR at any time point between LIPID and LIPID+CAR (P=0.897) indicating that L-carnitine infusion did not alter lipid-induced insulin resistance (Fig 2B). As a result, peripheral insulin sensitivity, expressed as the M-value, was blunted during the lipid infusion compared to the control condition (26.0±3.1 vs. 52.5±3.8 µmol/kg/min, P=0.019 respectively). Lipid-induced insulin resistance was not alleviated by L-carnitine infusion (M-value LIPID+CAR; 25.3±4.0 µmol/kg/min, P>0.99 compared to LIPID, Fig 2C).

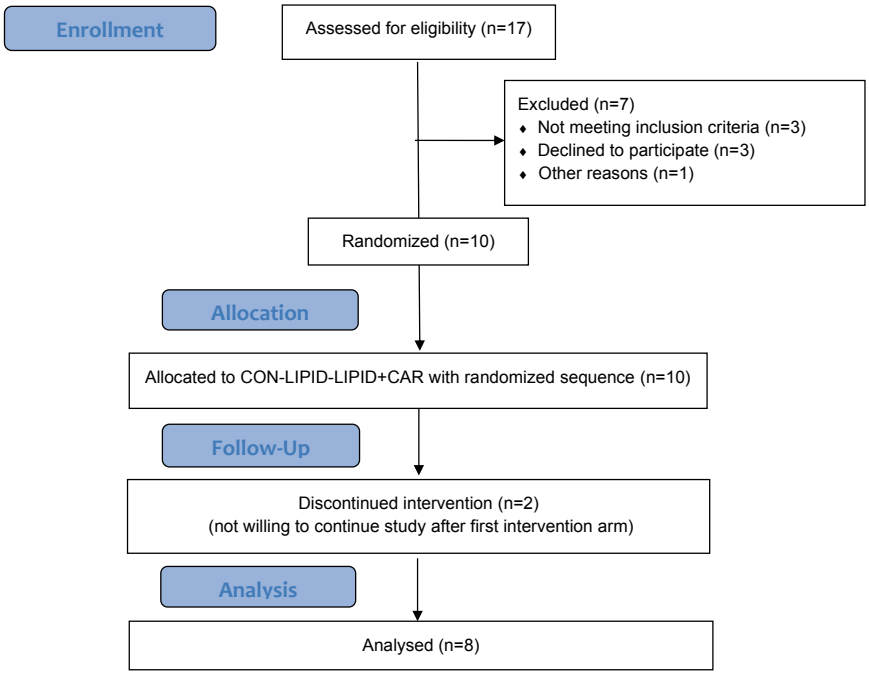

Figure 1. CONSORT flow diagram. Diagram of the progress through the phases of this randomized, controlled crossover study with young lean male participants.

Table 1. Baseline participant characteristics.

	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_{2max} (ml/min/kg bw)	42.4	±	2.5
W_{max} (W/kg bw)	3.3	±	0.2
Glucose metabolism			
Fasting glucose (mmol/L)	4.9	±	0.1
Fasting insulin (pmol/L)	28.9	±	3.9
HbA_{1c} (%)	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 ± S.E.M. W_{max}, maximal workload, VO_{2max} is normalized to body weight in kg

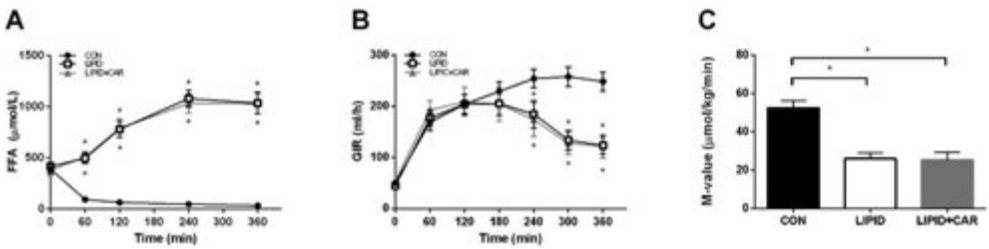

Figure 2. Hyperinsulinemic-euglycemic clamp. Plasma FFA concentrations (A), glucose infusion rates (B) and insulin sensitivity expressed as M-value (C) in CON, LIPID and LIPID+CAR during the hyperinsulinemic-euglycemic clamp (n=8). Data are expressed as mean ± SEM. *, Significantly different from CON (P<0.05).

Metabolic flexibility and substrate oxidation

Basal glucose oxidation were comparable between study arms but glucose oxidation upon 6 hours of insulin infusion was increased in the control condition (from 5.4±1.8 to 19.6±1.5 µmol/kg/min, P<0.01, Fig 3A). However, glucose oxidation remained low during the infusion of lipid (from 6.5±1.5 to 8.5±1.6 µmol/kg/min, P=0.164, Fig 3A). Parallel infusion of L-carnitine did not change the lipid-induced suppression in glucose oxidation (P=0.864). In line with these findings, lipid oxidation was comparable between study arms at baseline but was elevated after 6-hours of lipid infusion and suppressed in the control arm (1.8±2.7 and 0.6±0.2 µmol/kg/min in LIPID and CON respectively, P<0.01, Fig 3B). Also here, L-carnitine infusion did not affect lipid oxidation rates (P=0.883, table 2). The RER measured under baseline conditions, so before the start of the infusions, was not different between study arms, as expected (P=0.881, Fig 3C). Metabolic flexibility, expressed as $\Delta \mathrm{RER}_{\mathrm{clamp-basal}}$, was decreased upon lipid infusion compared to control (0.10±0.02 and 0.01±0.01 in CON and LIPID respectively, P<0.01, Fig 3D). L-carnitine did not change the lipid-induced decrease in metabolic flexibility (0.01±0.01 in LIPID+CAR, P=0.920).

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]
Δ clamp-basal	0.10 ± 0.02	0.01 ± 0.01[a]	0.01 ± 0.01[a]
CHO oxidation (µmol/kg/min)			
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]
Δ clamp-basal	9.5 ± 2.2	1.4 ± 0.8[a]	1.6 ± 1.2[a]
Lipid oxidation (µmol/kg/min)			
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[a]	1.8 ± 0.2[a]
Δ clamp-basal	-1.01 ± 0.11	0.18 ± 0.08[a]	0.19 ± 0.15[a]
M-value (µmol/kg/min)	52.5 ± 3.8	26.0 ± 3.1[a]	25.3 ± 4.0[a]

Data are expressed as mean ± S.E.M. (n=8), [a] significantly different from CON.

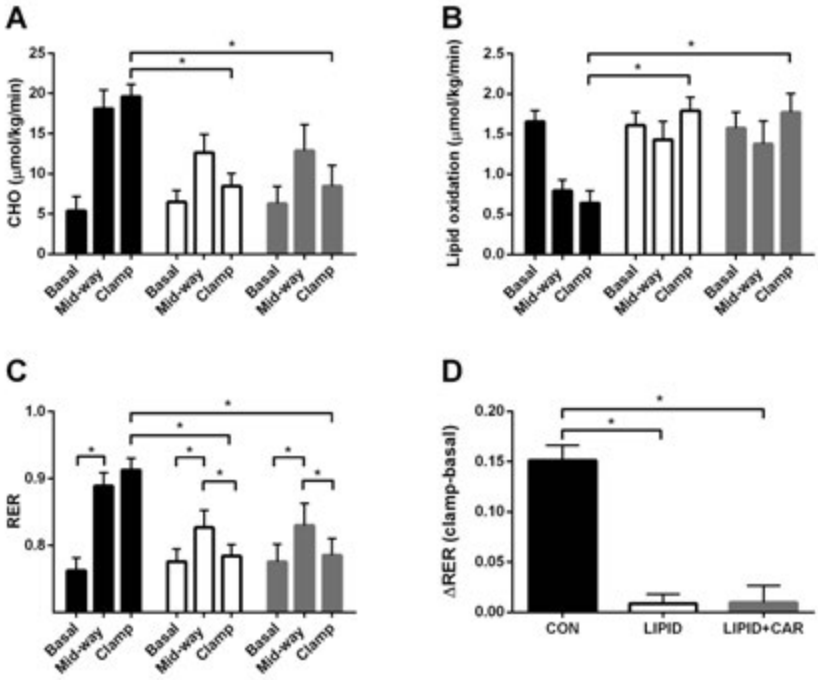

Figure 3. Metabolic flexibility and substrate oxidation. Glucose oxidation (A), Lipid oxidation (B), respiratory exchange ratio (RER) (C) and metabolic flexibility expressed as delta RER (D) assessed during a hyperinsulinemic-euglycemic clamp in CON, LIPID and LIPID+CAR (n=8). Black bars represent CON, 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).

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 vs. 36.3±2.8 vs. 34.4±1.9 µmol/L in CON, LIPID, LIPID+CAR respectively, P=0.829, Fig 4A and Table 3). One hour of L-carnitine infusion already increased plasma free carnitine availability to supra-physiological concentrations (155±5 µmol/L, P<0.01) and finally reaching concentrations of 183±6 µmol/L (P<0.01) after six hours of infusion (Fig 4A and Table 3). No changes from baseline in plasma free carnitine availability were observed in the CON and LIPID trial over time (P>0.99, Fig 4A and Table 3). Plasma acetylcarnitine (C2) concentrations showed a time*treatment interaction (P<0.01) and were comparable between study arms at baseline (P=0.860). C2 concentrations decreased in the CON trial over time from 5.4±0.7 to 3.6±0 µmol/L after one hour and to 2.3±0.1 µmol/L after six hours (P<0.01, Fig 4B and Table 3), which is probably due to insulin

infusion. With lipid infusion, C2 concentrations decreased during the first hour (5.7±0.6 to 4.2±0.4 μmol/L, P<0.01) and subsequently tended to increase again (P=0.05 compared to t=60), reaching concentrations of 6.3±0.7 μmol/L after 6 hours which are comparable to baseline values (P=0.375). Infusion of L-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, Fig 4B and Table 3). C3 and short-chain acylcarnitines (C3 until C5) both showed a time*treatment interaction (P<0.01) 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), but were increased upon 6 hours L-carnitine infusion compared to the CON and LIPID condition (P<0.01, Fig 4C and Table 3). 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 in the basal state 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<0.01). In contrast, medium- and long-chain acylcarnitines increased upon lipid infusion after six hours (P=0.034 and P=0.013 respectively, Fig 4D and 4E and Table 3). This increase was even more pronounced when combining lipid infusion with L-carnitine infusion for both medium- as long-chain acylcarnitines (P<0.01, Fig 4D and 4E and Table 3).

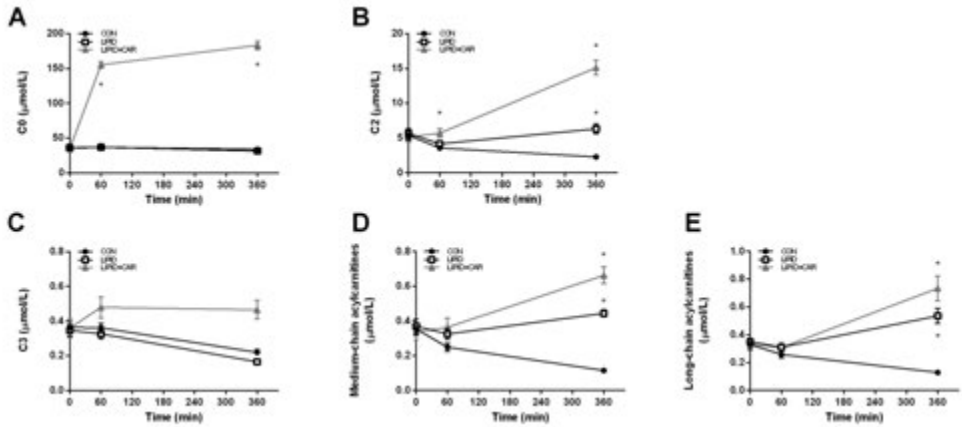

Figure 4. Plasma acylcarnitine profiles. Plasma free carnitine availability (A), acetylcarnitine (B), C_3 acylcarnitine (C), medium chain acylcarnitines (D) and long-chain acylcarnitine concentrations (E) measured at baseline (t=0), after one hour (t=60) and at the of the 6-hour hyperinsulinemic-euglycemic clamp (t=360) (n=8). Black dots represent the control group, white dots the LIPID group and light grey lines the LIPID+CAR group. Data are expressed as means ± SEM. * significantly different from CON ($P<0.05$). Note: in A the lines of the control and lipid conditions are overlapping.

Table 3. Plasma acylcarnitine concentrations before, during and after 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	±	$4.94^{a,b,c}$
	t=360	33.78	±	2.04	31.55	±	2.16	183.33	±	$6.28^{a,b,c,d}$
C2	t=0	5.38	±	0.71	5.65	±	0.65	5.13	±	0.65
	t=60	3.56	±	0.34^{c}	4.16	±	0.37^{c}	5.70	±	$0.62^{a,b}$
	t=360	2.27	±	$0.10^{c,d}$	6.31	±	$0.69^{a,d}$	15.11	±	$1.04^{a,b,c,d}$
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	±	$0.06^{a,b,c}$
	t=360	0.22	±	$0.02^{c,d}$	0.17	±	$0.01^{a,c,d}$	0.47	±	$0.05^{a,b,c}$
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	±	$0.02^{b,c}$
	t=360	0.15	±	$0.01^{c,d}$	0.14	±	$0.01^{c,d}$	0.28	±	$0.03^{a,b,c,d}$
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^{c}	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	±	$0.01^{a,b,c}$
	t=360	0.05	±	$0.01^{c,d}$	0.04	±	$0.01^{c,d}$	0.06	±	$0.01^{b,c,d}$
C4-3OH	t=0	0.03	±	0.00	0.04	±	0.01	0.04	±	0.01
	t=60	0.02	±	0.00^{c}	0.03	±	0.00^{c}	0.05	±	0.02
	t=360	0.01	±	$0.00^{c,d}$	0.06	±	$0.01^{a,c,d}$	0.14	±	$0.03^{a,b,c,d}$
C6	t=0	0.04	±	0.00	0.04	±	0.00	0.03	±	0.00
	t=60	0.03	±	0.00^{c}	0.03	±	0.00^{c}	0.04	±	0.01^{a}
	t=360	0.02	±	$0.00^{c,d}$	0.04	±	$0.00^{a,c,d}$	0.08	±	$0.01^{a,b,c,d}$

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[d]	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[c]	0.07	±	0.01[c]	0.08	±	0.01
	t=360	0.03	±	0.00[c,d]	0.07	±	0.01[a]	0.10	±	0.01[a,b,c,d]
C3 DC	t=0	0.03	±	0.00	0.04	±	0.00	0.04	±	0.01
	t=60	0.03	±	0.00[c]	0.03	±	0.00[a,c]	0.03	±	0.00[c]
	t=360	0.01	±	0.00[c,d]	0.03	±	0.00[a]	0.04	±	0.00[a,d]
C10:1	t=0	0.06	±	0.01	0.06	±	0.01	0.05	±	0.01
	t=60	0.04	±	0.01[c]	0.09	±	0.01[a,c]	0.10	±	0.02[a,c]
	t=360	0.01	±	0.00[c,d]	0.19	±	0.01[a,c,d]	0.27	±	0.02[a,b,c,d]
C10	t=0	0.07	±	0.01	0.08	±	0.01	0.07	±	0.01[c]
	t=60	0.05	±	0.01[c]	0.05	±	0.01[c]	0.06	±	0.01
	t=360	0.01	±	0.00[c,d]	0.04	±	0.00[a,c]	0.06	±	0.01[a,b]
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[c]	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[a]
	t=360	0.02	±	0.00[c]	0.02	±	0.00[a]	0.03	±	0.00[a]
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[c]	0.02	±	0.00[c]
	t=360	0.00	±	0.00[c,d]	0.03	±	0.00[a,d]	0.05	±	0.00[a,b,d]
C12	t=0	0.03	±	0.00	0.03	±	0.00	0.03	±	0.01
	t=60	0.02	±	0.00[c]	0.02	±	0.00[c]	0.03	±	0.00[c]
	t=360	0.01	±	0.00[c,d]	0.02	±	0.00[a,c]	0.03	±	0.00[a,b,d]
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[a]	0.02	±	0.00[a,b,c,d]
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[a]	0.02	±	0.00[a,b,c,d]
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
C14:2	t=0	0.02	±	0.00	0.02	±	0.00	0.02	±	0.00
	t=60	0.01	±	0.00[c]	0.03	±	0.00[a,c]	0.03	±	0.00[a,c]
	t=360	0.00	±	0.00[c,d]	0.12	±	0.02[a,c,d]	0.21	±	0.04[a,b,c,d]
C14:1	t=0	0.04	±	0.01	0.04	±	0.01	0.04	±	0.01
	t=60	0.03	±	0.00[c]	0.03	±	0.00[c]	0.03	±	0.01
	t=360	0.01	±	0.00[c,d]	0.06	±	0.01[a,d]	0.10	±	0.02[a,b,c,d]
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[c]	0.01	±	0.00[c]
	t=360	0.01	±	0.00[c,d]	0.01	±	0.00[a.c]	0.02	±	0.00[a,b]
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[a,c,d]	0.01	±	0.00[a,c,d]
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[a]	0.01	±	0.00[a]
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[c]	0.00	±	0.00
	t=360	0.00	±	0.00	0.00	±	0.00[c]	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[c]	0.01	±	0.00[c]	0.01	±	0.00
	t=360	0.00	±	0.00[c,d]	0.02	±	0.00[a]	0.02	±	0.00[a]
C16	t=0	0.08	±	0.01	0.08	±	0.00	0.08	±	0.01
	t=60	0.07	±	0.01[c]	0.08	±	0.00	0.07	±	0.01[c]
	t=360	0.03	±	0.00[c,d]	0.07	±	0.00[a,c,d]	0.08	±	0.01[a,b]
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[c]	0.04	±	0.00[a,c]	0.04	±	0.00[a,c]
	t=360	0.02	±	0.00[c,d]	0.13	±	0.01[a,c,d]	0.15	±	0.02[a,b,c,d]
C18:1	t=0	0.08	±	0.01	0.10	±	0.01	0.09	±	0.01
	t=60	0.06	±	0.01[c]	0.07	±	0.01[c]	0.07	±	0.01[c]
	t=360	0.04	±	0.00[c,d]	0.08	±	0.01[a]	0.09	±	0.00[a,b,c,d]
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[c,d]	0.03	±	0.00[a]	0.03	±	0.00[a]
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[c,d]
	t=360	0.00	±	0.00	0.01	±	0.00[a,c]	0.01	±	0.00[a]
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[a,c,d]
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
Short-chain	t=0	0.80	±	0.06	0.78	±	0.06	0.79	±	0.07
	t=60	0.76	±	0.05	0.72	±	0.05	0.97	±	0.08[a,b,c]
	t=360	0.50	±	0.04[c,d]	0.51	±	0.03[c,d]	1.06	±	0.08[a,b,c]
Medium-chain	t=0	0.35	±	0.04	0.37	±	0.04	0.34	±	0.05
	t=60	0.25	±	0.02[c]	0.33	±	0.03[a]	0.36	±	0.05[a]
	t=360	0.12	±	0.00[c,d]	0.44	±	0.02[c,d]	0.66	±	0.05[c,d]
Long-chain	t=0	0.33	±	0.04	0.35	±	0.02	0.33	±	0.04
	t=60	0.26	±	0.03[c]	0.31	±	0.02[a,c]	0.31	±	0.04[a]
	t=360	0.13	±	0.01[c,d]	0.53	±	0.05[a,c,d]	0.74	±	0.09[a,b,c,d]

Data are expressed as mean ± SEM, [a] significantly different from CON, [b] significantly different from LIPID, [c] significantly different from t=0, [d] significantly different from t=60.

Skeletal muscle acylcarnitines profiles

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 (Fig 5A and 5B, table 4). Because of Bonferroni correction for multiple testing, p-values of 0.0125 were considered statistically significant for the comparison of insulin-stimulated states with the basal state. Free carnitine and acetylcarnitine values obtained after 6 hours were not different from baseline (C0: P=0.734 (CON), P=0.170 (LIPID), P=0.192 (LIPID+CAR) and C2: P=0.138 (CON), P=0.368 (LIPID), P=0.187 (LIPID+CAR)). Short-chain acylcarnitine species reduced upon 6-hours of insulin infusion in CON trial compared to basal (P=0.013). This decrease was blunted upon LIPID and LIPID+CARN compared to CON, resulting in a tendency towards higher short-chain acylcarnitine concentrations upon LIPID and LIPID+CARN compared to CON (P=0.103, Fig 5C and table 4). 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 (P=0.049 and P=0.107 respectively, Fig 5D and E). No difference in medium and long-chain acylcarnitines were found after 6-hours of infusion between groups (P=0.177 and P=0.564 respectively, Figure 5D and E and table 4)

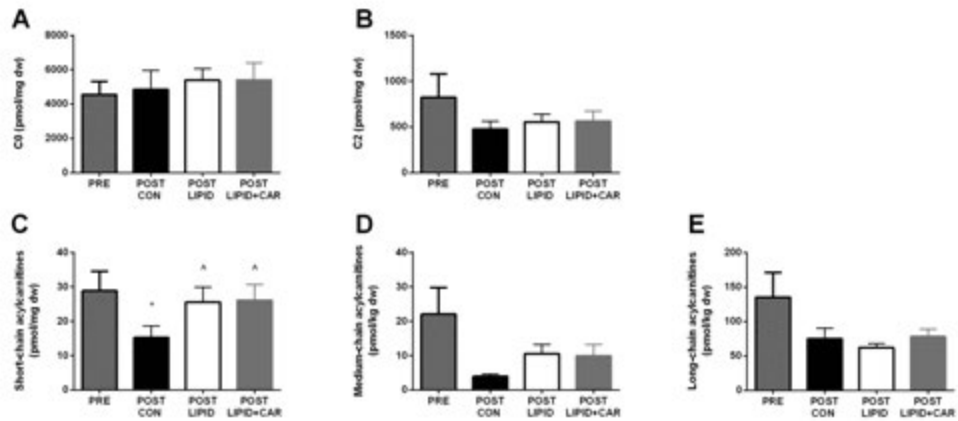

Figure 5. Skeletal muscle acylcarnitine profiles. Skeletal muscle acylcarnitine concentrations measured in biopsies at the end of the 6-hour hyperinsulinemic-euglycemic clamp (n=8). Black bars represent the control group, white bars the LIPID group and light grey bars the LIPID+CAR group. Dark grey bars represent the pre-clamp muscle biopsy. Data are expressed as means ± SEM. * significantly different from PRE (P<0.0125 after Bonferroni correction for multiple testing), ^ tending to be different from CON (P<0.10).

Table 4. 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[a]	6.26	±	2.80	10.39	±	2.90[a]
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[a,c]	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[b]
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[a]	0.18	±	0.06	0.15	±	0.03[a]
Short-acylcarnitines	16.04	±	3.43	29.38	±	4.48[b]	28.88	±	5.69	29.73	±	5.52[b]
Medium-acylcarnitine	3.17	±	0.60	6.83	±	1.47	22.07	±	7.70	6.38	±	1.78
Long-acylcarnitines	74.97	±	15.53	61.71	±	6.19	135.09	±	36.20	77.59	±	11.32

Data are expressed as mean ± SEM, [a] significantly different from CON after, [b] tending to be different from CON after, [c] significantly different from LIPID+CAR before.

DISCUSSION

In the present study, we aimed to investigate whether free carnitine availability could alleviate lipid-induced insulin resistance. We hypothesized that intravenous infusion of L-carnitine would increase the availability of free carnitine in skeletal muscle, which could prevent the development of lipid-induced insulin resistance and metabolic inflexibility during acute lipid infusion.

In the current study, the intravenous administration of L-carnitine increased plasma free carnitine concentrations to 183 μmol/L. These values exceed normal reference values (22.3-54.8 μmol/L) indicating a state of hypercarnitinemia in the plasma and thus increased plasma free carnitine availability. This level of plasma hypercarnitinemia is comparable to earlier studies that also used L-carnitine infusions of similar dosage to reach hypercarnitinemia in the plasma (21, 35).

Although plasma hypercarnitinemia occurred, no differences in skeletal muscle free carnitine concentration were found upon L-carnitine infusion. This is surprising, as the infusion of insulin has been shown to stimulate uptake of carnitine and combinations of carnitine and insulin have been shown to increase carnitine content in muscle (36). 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 (36-40). Inhibition of the 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 (39, 41). Insulin is known to facilitate carnitine uptake into skeletal muscle by increasing the activity of the sarcolemmal Na^+/K^+ ATPase pump (36, 38, 42). Therefore, L-carnitine infusion together with hyperinsulinemia ($40mU/m^2/min$) was expected to increase skeletal muscle free carnitine levels, however, this was not the case in the present study. It is yet unclear why carnitine concentrations did not increase in muscle tissue. Although this very much exceeds the scope of the current research, a possible theoretical 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 blunted, thereby not leading to enhanced sodium mediated co-transport of carnitine into the skeletal muscle (38, 39, 43-45). However, this remains speculation and future studies will have to investigate

potential mechanisms. Unfortunately, we did not perform a clamp with intravenous infusion of L-carnitine, without additional lipid infusion. The latter could have revealed whether lipid infusion indeed hampered carnitine uptake versus insulin infusion alone. Furthermore, the participants of the current study were young and healthy and it is expected that their carnitine availability in muscle was already high to start with. It is conceivable that therefore an increase in muscle carnitine concentration upon infusion is less likely, although this requires further study.

The increase in lipid availability as a result of lipid infusion lead to strongly elevated plasma free fatty acid levels, as reported before (3, 34). 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, 34, 46-48). 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 L-carnitine. As carnitine needs to be taken up in the muscle to exert an effect on insulin sensitivity according to our hypothesis, it is not be surprising that insulin sensitivity was not affected in the present study. In contrast to our findings, beneficial effects of L-carnitine infusion has been reported previously in overweight patients with type 2 diabetes. Thus, in these studies, intravenous infusion of L-carnitine during a hyperinsulinemic-euglycemic clamp (40mU/kg/min) was shown to improve whole-body glucose disposal (20, 21). Furthermore, Mingrone et al. (20) reported enhanced insulin stimulated glucose oxidation upon L-carnitine infusion during a clamp (40mU/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 therefore, no lipid-induced insulin resistance occurred and the uptake of carnitine may have been more efficiently stimulated by insulin. Whether improved skeletal muscle free carnitine availability indeed underlies the beneficial metabolic effects that were reported previously, remains to be shown. 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. These decreases in acylcarnitine species are likely to reflect a decreased lipid oxidation caused by hyperinsulinemia, as previously reported (49, 50). 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 (51, 52). The main contributor to the plasma acetylcarnitine elevations might be increased production by 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 (52, 53). 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 branched-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 suggests that carnitine infusion can further stimulate the efflux of β-oxidation intermediates from the liver.

Surprisingly, skeletal muscle acetylcarnitine concentrations remained unaffected by lipid infusion as well as lipid combined with L-carnitine infusion. Contrary, Tsintzas et al. (48) 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 what is underlying this difference.

Furthermore, skeletal muscle short-chain acylcarnitine (C3-C5) 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 decrease in skeletal muscle acylcarnitine species upon insulin therefore probably reflects this decreased FFA availability resulting in a transition of lipid towards glucose

oxidation induced by hyperinsulinemia (49, 50). Lipid infusion increased plasma FFA concentrations despite high insulin concentration. Therefore, the decrease in short- and medium-chain acylcarnitines in skeletal muscle tissue as found in the control trial upon insulin was blunted upon the combination of lipid and insulin infusion, which may indicate higher skeletal muscle lipid oxidation rates upon the elevation of plasma lipids by lipid infusion. Remarkably, this effect was only seen on the short and medium chain acylcarnitine species and not on the long chain species: lipid infusion did not blunt the insulin-induced reduction in long-chain acylcarnitine species. Although we cannot provide a direct explanation for this effect, it could be speculated that during acute lipid overload, accumulation of β-oxidation intermediates does mainly happen at later passages through the β-oxidation.

A study limitation is the low number of participants in the current study. Although the number of participants is quite low (n = 8), a cross-over design was used to bolster the power of this mechanistic study. According to our sample size calculation, 13 participants needed to perform the entire study. Upon eight finalized participants, an interim analysis was performed. We calculated that based on the difference found in insulin sensitivity in the current study (eight participants), over five hundred participants would have been needed to render the effect of carnitine supplementation on insulin sensitivity statistically significant. Considering the reproducibility of the methods used, the sample size of n=8 should be sufficient to be able to pick up a change in insulin sensitivity of 10%, which is assumed to be clinically relevant. Thus, it can be inferred from the observed results that if there is a difference between treatments, it is so small that it is not clinically relevant. Therefore, the study was ended prematurely after eight participants. A second limitation of the current study is the fact that we only included men. The hormonal changes that occur in women as result of the menstrual cycle are known to affect insulin sensitivity (54, 55) and would have had to be taken into account. This would have made the execution of the current study with a three arm cross-over design very complex.

In summary and conclusion, lipid infusion strongly increased plasma FFA levels and resulted in a hampered metabolic flexibility and insulin sensitivity. The addition of intravenous infusion of L-carnitine elevated plasma free carnitine availability as expected. However, against expectations, L-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. Since skeletal muscle free carnitine availability remained unaltered with L-carnitine infusion, we cannot conclude on the original hypothesis whether free carnitine availability in skeletal muscle tissue might be crucial in maintaining metabolic flexibility and insulin sensitivity. Therefore, further research is necessary to unravel if skeletal muscle free carnitine availability is indeed crucial in maintaining metabolic flexibility and insulin sensitivity. Using an acute carnitine treatment, the current study was performed to mechanistically investigate the role of L-carnitine. However, to investigate whether carnitine treatment would be beneficial in improving insulin sensitivity in clinical practice, future studies using long-term carnitine treatment needs to be performed.

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Author contribution
Y.M.H.B. designed and performed the experiments, analyzed the data, and wrote the manuscript; Y.J.M.K., B.H., G.S., E.K., assisted during the experiments and reviewed and edited the manuscript. E.P., L.L. contributed to the design of the study, assisted during the experiments and reviewed and edited the manuscript. J.E.W., M.K.C.H., P.S., and V.B.S contributed to the design of the study, interpretation of the data, and reviewed and edited the manuscript. All authors approved the final version of the manuscript. V.B.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Disclosures
None of the authors has relevant conflict of interest to disclose.

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Chapter 5

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

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ABSTRACT

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

Methods: Thirty-two patients followed a 12-week L-carnitine treatment (2970 mg/day, orally). Insulin sensitivity was assessed by a two-step hyperinsulinemic-euglycemic clamp. *In vivo* skeletal muscle acetylcarnitine concentrations at rest and post exercise (30 minutes, 70% W_{max}), and intrahepatic lipid content (IHL) were determined by proton magnetic resonance spectroscopy (^{1}H-MRS). All measurements were performed before and after 12-weeks carnitine supplementation.

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

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

Trial registration number (clinicaltrials.gov): NCT03230812

INTRODUCTION

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

Acetylcarnitine gained attention as an important metabolite in light of maintaining insulin sensitivity and glucose homeostasis (3-6). In animal models, decreased skeletal muscle acetylcarnitine concentrations are reported in obese insulin-resistant mice, along with blunted metabolic flexibility and elevated plasma glucose levels (5), where metabolic flexibility is defined as the ability to switch from predominantly fat oxidation while fasted towards carbohydrate oxidation upon insulin stimulation (2). We previously developed a novel non-invasive proton magnetic resonance spectroscopy (^{1}H-MRS) method to enable determination of *in vivo* skeletal muscle acetylcarnitine concentrations in humans (3). Applying this technique, we showed that insulin sensitivity correlates positively with *in vivo* magnetic resonance (MR)-based skeletal muscle acetylcarnitine concentration (3), indicating lower acetylcarnitine levels in muscle of insulin resistant individuals and T2DM patients.

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

We recently showed that carnitine supplementation in pre-diabetic individuals elevated MR-based skeletal muscle acetylcarnitine levels and improved metabolic flexibility (9). However, insulin sensitivity remained unchanged in these individuals, which could be attributed to the short supplementation period of 4 weeks (10). In T2DM patients, positive results of oral carnitine supplemention have been reported on fasting plasma glucose (FPG) (11-14), fasting insulin (12, 14), HbA_{1c} (14) and markers of insulin sensitivity as assessed by Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) (10-12, 14). These results point towards improved insulin sensitivity by carnitine in T2DM, but this has never been investigated using the gold standard 2-step hyperinsulinemic-euglycemic clamp procedure, which evaluates both hepatic and peripheral insulin sensitivity. Furthermore, the mechanism underlying improved insulin sensitivity in humans remains elusive and it is unclear whether an increased formation of acetylcarnitine may explain such improvements. We here hypothesize that the positive effects of carnitine on insulin sensitivity and glucose homeostasis in T2DM depend on the improvement of the capacity to form acetylcarnitine. Therefore, we here investigated if carnitine supplementation for 3 months elevates skeletal muscle acetylcarnitine while also improving insulin sensitivity and glucose homeostasis in T2DM patients. We also investigated whether the acetylcarnitine concentrations prior to the start of the study are predictive for the response to the supplementation. As an explorative objective, we also investigated whether (potential) improvements in insulin sensitivity by carnitine translate into benefits in daily life functioning (i.e. physical and cognitive performance) since previous research indicated beneficial effects on these parameters after carnitine supplementation (15).

METHODS

Ethical Approval

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

Participants

Thirty-two overweight/obese (BMI: 25-38 kg/m^2), middle aged (40-75 years) T2DM patients were included in this study. Both male and post-menopausal female T2DM patients were included. T2DM patients were either on diet (n=5), metformin (n=15), sulfonylurea (n=3) or metformin + sulfonylurea (n=9) therapy for at least 6 months prior to the start of the study. At screening, exclusion criteria were assessed and evaluated by a medical doctor. Exclusion criteria were the use of insulin therapy, uncontrolled diabetes (HbA_{1c} > 9.5%), clinically relevant diabetes-related complications (i.e. active diabetic foot, polyneuropathy or retinopathy), uncontrolled hypertension (blood pressure measurement), medical history or active cardiovascular disease (Electrocardiogram, ECG), impaired kidney and/or renal function (plasma ASAT, ALAT, GGT, creatine and bilirubin levels), anemia (Hb <7.8 mmol/L), MRI contra-indications, unstable body weight (weight gain or loss >5 kg in the previous 3 months), engagement in more than 3 hours of exercise a week, and being vegetarian (because of altered whole body carnitine status).

Experimental Design

The study was set up as an intervention trial was performed between March 2018 and November 2019. All patients underwent baseline measurements (visits 1, 2, 3 and 4, Figure 1) followed by 3 months (96 days) of oral carnitine supplemention (next to the participants usual medication). Between day 90-96 of the carnitine supplementation period, measurements were repeated (visit 7, 8 and 9, Figure 1). Compliance was checked during a monthly visit by counting unused supplements and furthermore by measuring plasma free carnitine levels at baseline and after 3 months. Participants were asked to maintain their usual physical activity pattern and regular diet during participation in the study. Changes in physical activity level were monitored by Baecke's habitual physical activity questionnaire at baseline and 3 months (16). To prevent

additional intake of oral carnitine, consumption of food supplements was not allowed. Participants refrained from alcohol and strenuous physical activity three days prior to a testday.

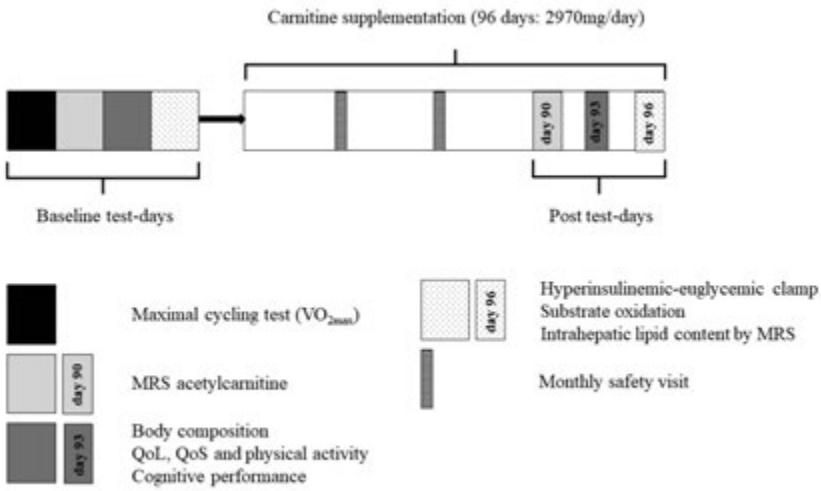

Figure 1. Study outline of the 96-day carnitine supplementation interventional trial in type 2 diabetes patients. Participants performed four baseline test-days followed by 3 months (equal to 96 days) of oral carnitine supplementation. Post test-day were conducted during the last week of supplementation (day 90, 93 and 96). MRS, magenetic resonance spectroscopy; QoL, quality of life; QoS, quality of sleep. For one participant the supplementation period was prolonged due to personal circumstances (99 days), all other participants adhered to the 96-day schedule.

Treatment

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

Outcomes

The primary study endpoint was peripheral insulin sensitivity (expressed as ΔR_d), as

measured by a hyperinsulinemic-euglycemic clamp. Other endpoints were acetylcarnitine concentrations before and after exercise, metabolic flexibility, intrahepatic lipid content, body composition, plasma metabolites (i.e. glucose, HbA_{1c} and acylcarnitines), and patients perceived benefits in general daily functioning (i.e. physical and cognitive performance, quality of life and quality of sleep).

VO_{2max}

For characterization (visit 1), participants performed a graded cycling test to determine maximal oxygen uptake (VO_{2max}) and maximal power output (W_{max}) as previously described (18).

^{1}H-MRS acetylcarnitine

At visit 2 and 7 (day 90), participants consumed a light lunch at noon (12:00 PM) and remained fasted for the following 5 hours. Participants refrained from physical activity and reported to the laboratory at 4:30 PM. After arrival, subjects rested for 30 minutes and at 5:00 PM, resting skeletal muscle acetylcarnitine concentrations were assessed by ^{1}H-MRS. Subsequently, as high intensity exercise is known to stimulate the formation of acetylcarnitine (19, 20), participants performed a 30-minute cycling exercise at 70% of the participants' predetermined maximal power output (W_{max}). Directly after cycling, skeletal muscle acetylcarnitine concentrations were quantified again. Skeletal muscle acetylcarnitine concentrations were determined *in vivo* as previously validated and reported on a 3T clinical MR scanner (Achieva 3T-X, Philips Healthcare, Best, The Netherlands) (21). Spectra were analysed with a custom-made MATLAB script (The Mathworks Inc., Natrick, USA) and acetylcarnitine concentrations were calculated as previously described (3).

Body composition

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

Physical performance

In the late morning (10.30 AM) of visit 3 and 8, two physical performance tests were conducted: a Timed Chair-Stand Test (TCST) and a 6-Minute Walk Test (6MWT).

During the TCST, participants performed 10 chair-rise repetitions as fast as possible, while performance time was recorded. For the 6MWT, participants were instructed to walk (no running) as far as possible within 6 minutes on a 10m flat track.

Quality of Sleep and Quality of Life

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

Cognitive Performance

Cognitive performance was assessed at visit 3 and 8 using the Cambridge neuropsychological test automated battery (CANTAB). All participants performed the CANTAB battery in a quiet chamber in the fasting state. Three different cognitive domains were investigated: psychomotor speed (selecting specific information), executive function (planning, decision-making and impulse control) and memory (ability to store and retrieve information). Psychomotor speed was determined using the motor screening task (MOT) and reaction time (RT). Executive function was assed via multitasking tests (MTT) and spatial span (SSP), while delayed matching to sample (DMS) and paired associates learning (PAL) were used to investigate memory. Data analysis of MOT, RT, MTT, SSP, DMS and PAL was performed as previously described (25).

Hyperinsulinemic-Euglycemic two-step clamp

At visit 4 and 9, all participants came to the univerisity (06:00 AM) after an overnight fast and underwent a two-step hyperinsulinemic-euglycemic clamp with co-infusion of D-[6,6-$^2H_2$] glucose tracer (16.8 mg/mL, 0.04 ml/kg/min) to determine insulin sensitivity as previously described (26). After 180 min of glucose tracer infusion (sufficient time to reach an equilibrium in type 2 diabetes patients), a low dose insulin infusion (10 mU/m^2/min) was started for 3 hours to assess hepatic insulin sensitivity, followed by 2.5 hours of high dose insulin infusion (40 mU/m^2/min) to measure peripheral insulin sensitivity. During the steady states (t=150-180 min, t=330-360 min,

and t=480-510 min) blood samples were collected every 10 minutes (4 times per steady state) and substrate utilization was assessed via indirect calorimetry using a ventilated hood (Omnical, IDEE, Maastricht, The Netherlands) and calculated according to Frayn et al. (27) (assuming 15% protein oxidation). The respiratory exchange ratio (RER), defined as VCO_2/VO_2, was used to determine metabolic flexibility (ΔRER). Metabolic flexibility (ΔRER) reflects the difference between the insulin stimulated RER minus RER at basal conditions. Isotopic enrichment of plasma glucose was quantified by electron ionization gas chromatography-mass spectrometry as previously described (28). Steele's single pool non-steady state equations were used (to correct for potential small deviations from steady states) to calculate glucose appearance (R_a) and disappearance (R_d) (29). Volume of distribution was assumed to be 0.190 L/kg for glucose.

^{1}H-MRS intrahepatic lipid

At visit 4 and 9, proton magnetic resonance spectroscopy (^{1}H-MRS) was performed prior to the start of the insulin infusion of the hyperinsulinemic-euglycemic clamp (07:00 AM) to determine intrahepatic lipid content (IHL). IHL content was determined on a 3T clinical MR scanner (Achieva 3T-X, Philips Healthcare, Best, The Netherlands). A STEAM sequence was used to acquire spectra using the following acquisition paramaters: repition tme (TR)=4500 ms, echo time (TE)=20 ms, mixing time (TM)=16 ms, spectral bandwidth 2 kHz, number of acquired data points 2048, volume of interest (VOI)=30x30x30 mm and the number of signal averages (NSA)=128. VAPOR water suppression was applied and an additional water reference scan (NSA=16) was obtained. Spectra were post-processed in a home-written MATLAB (MATLAB 2014b, The MathWorks, Inc., Natick, Massachusetts, United States) script. Target (lipid-CH_2 and water) resonances were fitted individually in the respective spectrum. IHL was expressed in absolute (weight/weight) percentage using the ratio of T_2 corrected signal intensities of lipid-CH_2 and water.

Plasma metabolites

Plasma metabolites were analysed at baseline and after 3 months of carnitine supplementation. Acylcarnitine species were analysed via tandem mass spectrometry as previously described (30). Glucose (Horiba, Montpellier, France), free fatty acids (Wako Chemicals, Neuss, Germany), triglycerides (Sigma, St. Louis, USA) and cholesterol (Roche Diagnostics, Mannheim, Germany) concentrations were determined colorimetrically on the Cobas Pentra analyzer (Horiba, Montpellier, France). HbA_{1c}

levels were determined using the NU kit (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) Plasma and insulin levels via a human insulin Elisa kit (Crystal Chem, Elk Grove Village, USA).

Sample size calculation and statistics

The sample size was chosen in order to be able to detect a clinically significant improvement in insulin sensitivity of 8-15% as assessed during a hyperinsulinemic-euglycemic clamp (31, 32). Taking the intraindividual variation of the difference in insulin sensitivity in repeated measurements of 7-15% into account (33, 34), the chosen sample size of N=32 was amply sufficient (for β >80%, α <0.05, two-sided testing). Data are presented as means ± SEM and a p-values of <0.05 was considered significantly different (two-sided testing, α=0.05, 1-β of 80%). Statistical analysis were conducted using SPSS 24.0 software (IBM Corp: IBM SPSS statistics for Windows, Armonk New York, USA). Normal distribution of the data was evaluated using a Shapiro-Wilk normality test. Student paired sample t-tests were performed to investigate differences before and after 3 months of oral carnitine intake if data were normally distributed. In case data were not normally distributred, the non paramatric Wilcoxon signed-rank. To investigate whether acetylcarnitine concentrations before the intervention (or other characteristics such as age and BMI) were predictive of the response to the intervention, a multivariable linear regression analysis was performed.

RESULTS

Participant characteristics

Thirty-two participants with well-controlled T2DM (HbA_{1c} of 6.6% [49.0 mmol/mol] completed the study (21 men, 11 women). All participants were overweight to obese (BMI of 30.0 ± 1.0 kg/m²) with an average age of 65 ± 1 years. The total overview of the participant characteristics is illustrated in table S1. No dropouts or serious adverse events were reported. No major side effects of the oral carnitine supplementation were reported. Five participants experienced a slight fishy body odour, a recognized side effect of carnitine.

Study compliance

As a sign of compliance, plasma free carnitine (C0) as well as acetylcarnitine (C2) levels increased in all individuals upon carnitine supplementation ($p<0.01$ for both, figure 2A and B). All participants returned the surplus of carnitine chewing tablets and the compliance rate was calculated as the number of tablets ingested relative to the prescribed number. The compliance rate during the 3-month carnitine supplementation period was 97.1 ± 0.7%. In line with the instruction to maintain the usual life style during participation in the study, no differences were observed in physical activity level ($p=0.278$, table 1) and body mass ($p=0.248$) and composition (fat mass $p=0.909$, fat free mass $p=0.392$, table 1) after carnitine supplementation.

Carnitine supplementation increased peripheral and hepatic insulin sensitivity

Peripheral insulin sensitivity was markedly improved by 31% after carnitine supplementation ($p=0.005$, figure 2C and table 2). Elevation in insulin-stimulated non-oxidative glucose disposal ($\Delta NOGD_{high-basal}$) largely explained this increased peripheral insulin sensitivity ($p=0.004$, figure 2D and table 2), whereas the insulin-stimulated glucose oxidation ($\Delta CHO_{high-basal}$) remained unaffected by carnitine supplementation ($p=0.866$, Table 2). Absolute glucose oxidation tended to be higher in all stages of the hyperinsulinemic-euglycemic clamp after carnitine supplementation but only reached statistical significance in the low-insulin state ($p=0.018$, figure 2E and table 2). Lipid oxidation showed the reciprocal pattern and tended to be lower after carnitine supplementation in all stages of the clamp. Similarly, statistical significance was reached in the low-insulin state ($p=0.004$, figure 2F and table 2) while it remained a tendency towards significance during the high-insulin state ($p=0.063$, figure 2F and table 2). Metabolic flexibility was not different in the high

insulin phase (p=0.710) but improved in the low insulin phase upon carnitine supplementation (p=0.007, table 2). Interestingly, hepatic insulin sensitivity (ΔEGP suppression) strongly improved with 22% from 32.4 ± 3.0 to 39.5 ± 3.2%; p=0.020, figure 2G.

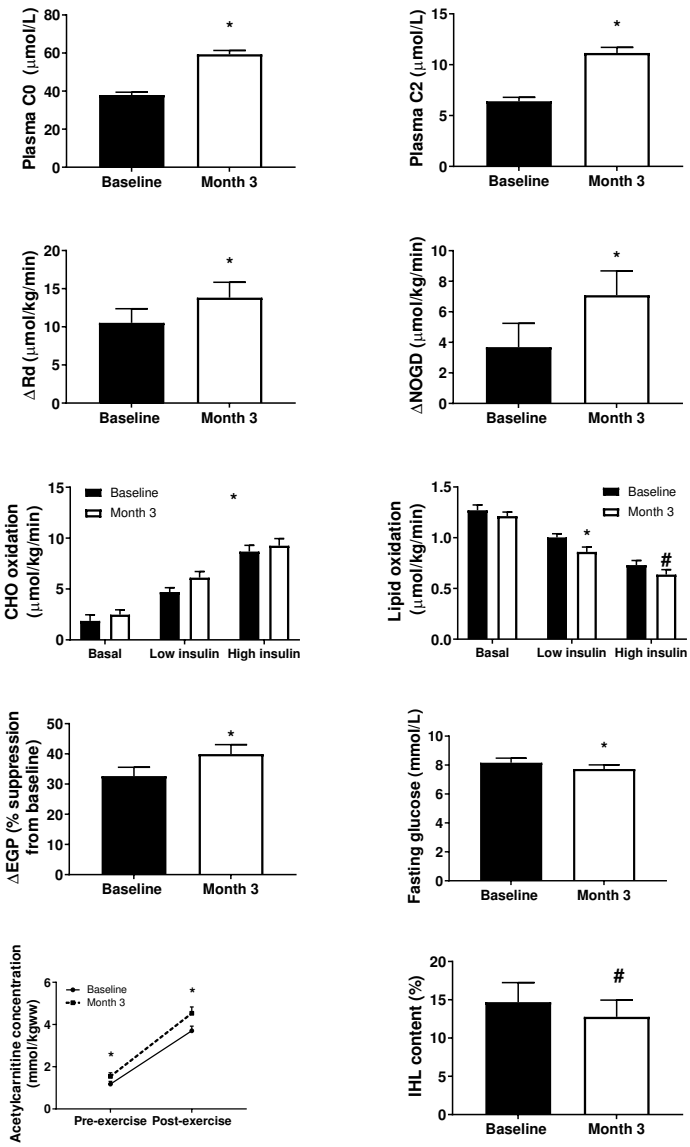

Figure 2

Figure 2. (Previous page.) Study compliance represented via plasma levels of free carnitine (A) and acetylcarnitine (B) concentrations (n=32). Plasma samples were obtained in the overnight fasted state at baseline and month 3. Change in peripheral insulin sensitivity (n=30) expressed as the insulin-stimulated rate of disappearance of glucose (ΔR_d) during 40 mU/m²/min of insulin infusion (C), insulin-stimulated non-oxidative glucose disposal (n=30) during 40 mU/m²/min of insulin infusion (D), substrate oxidation including carbohydrate (CHO) oxidation (E) and lipid oxidation (F), both n=30, the percentage suppression of the endogenous glucose production (ΔEGP) as marker for hepatic insulin sensitivity (n=31) with 10 mU/m²/min insulin infusion (G), and fasting plasma glucose (n=32) concentrations (H) are illustrate in the T2DM patients at baseline and month 3 after carnitine supplementation. MR-based skeletal muscle acetylcarnitine concentrations in the m. vastus lateralis in rest (pre-exercise) and the capacity to form acetylcarnitine with exercise (post-exercise) as marker of free carnitine availability (I) (n=32) and Intrahepatic lipid content (J) (n=31) determined using proton magnetic spectroscopy using a STEAM sequence are illustrated in type 2 diabetes patients before and after 3 months of carnitine supplementation. Data are presented as mean ± SEM and n=32. C0: free carnitine, C2: acetylcarnitine. * Significantly different ($p<0.05$), # trend towards a significant difference ($p<0.10$)

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

Variable	Baseline	Month 3	Delta [b]	P-value
Body weight (kg)	89.41 ± 2.80	89.78 ± 2.80	0.37 ± 0.32	0.248
Fat free mass (kg)	55.77 ± 1.75	56.12 ± 1.69	0.34 ± 0.40	0.392
Fat mass (kg)	33.63 ± 1.84	33.58 ± 1.96	-0.05 ± 0.45	0.909
Fat percentage (%)	37.20 ± 1.36	36.88 ± 1.42	-0.32 ± 0.48	0.517
Total physical activity score [a]	7.61 ± 0.26	7.73 ± 0.26	0.12 ± 0.11	0.278
Fasting glucose (mmol/L)	8.2 ± 0.3	7.7 ± 0.3	-0.43 ± 0.80	0.083
HbA1c (mmol/mol)	48.7 ± 1.5	48.0 ± 1.5	-0.75 ± 0.80	0.357
Insulin (μU/mL)	7.8 ± 8.39	7.5 ± 7.9	-3.12 ± 5.90	0.601
Free fatty acids (μmol/L)	627.9 ± 49.3	603.5 ± 47.4	-24.4 ± 22.7	0.299[c]
Triglycerides (mmol/L)	1.9 ± 0.2	1.9 ± 0.2	0.06 ± 0.07	0.413
Cholesterol (mmol/L)	4.2 ± 0.2	4.3 ± 0.2	0.10 ± 0.10	0.287

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

Fasting plasma glucose levels tended to be lower after carnitine supplementation

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

Enhanced skeletal muscle acetylcarnitine formation due to carnitine supplementation

To investigate whether improvements in skeletal muscle acetylcarnitine metabolism contributed to the enhanced peripheral insulin sensitivity and substrate oxidation after carnitine supplementation, acetylcarnitine concentrations were measured *in vivo* in the m. vastus lateralis using ^{1}H-MRS before and after exercise. Indeed, resting skeletal muscle acetylcarnitine concentration increased after carnitine supplementation (1.18 ± 0.13 vs. 1.54 ± 0.17 mmol/kgww; p=0.004, Figure 2I). Skeletal muscle acetylcarnitine levels were determined after 30 min of exercise at 70% W_{max}. Exercise is a strong stimulator of skeletal muscle acetylcarnitine formation (9, 19), possibly because mitochondrial substrate flux rapidly increases. This eventually leads to elevated intra-mitochondrial acetyl-CoA levels and concomitant increased acetylation of carnitine to acetylcarnitine. Therefore, at high exercise intensity, acetylcarnitine concentration strongly increases and the total pool of free carnitine is almost completely acetylated. Under these conditions, acetylcarnitine concentration therefore reflect free carnitine availability. Indeed, post-exercise acetylcarnitine concentrations increased after carnitine supplementation (3.70 ± 0.22 vs 4.53 ± 0.30 mmol/kgww; p<0.001, Figure 2I) suggesting carnitine supplementation to improve free carnitine availability. Furthermore, we investigated whether patient characteristics (such as age, BMI and carnitine status (high-low levels prior to the start of the study)) could be predictive for the individual improvement in insulin sensitivity in the current study. However, none of the investigated characteristics was associated with the response in insulin sensitivity in the current study (p>0.05).

Tendency towards reduced intrahepatic lipid content

Intrahepatic lipid content (IHL) was determined *in vivo* using ^{1}H-MRS to investigate if a reduction in IHL could have contributed to the improvement in hepatic insulin sensitivity. Interestingly, liver fat content tended to be reduced after carnitine supplementation (from 14.7 to 12.8 ± 2.2%; p=0.098, figure 2J).

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

	Baseline	Month 3	Delta [a]	P-value
R_d glucose (μmol/kg/min)				
Basal	11.73 ± 0.53	10.96 ± 0.53	-0.77 ± 0.50	0.136
10 mU/m²/min	10.51 ± 0.33	11.02 ± 0.50	0.51 ± 0.38	0.190
40 mU/m²/min	22.26 ± 1.62	24.80 ± 1.83	2.54 ± 1.05	0.022
Δ10-basal	-1.22 ± 0.52	0.05 ± 0.64	1.28 ± 0.61	0.046
Δ40-basal	10.53 ± 1.85	13.83 ± 2.02	3.31 ± 1.10	0.005
EGP (μmol/kg/min)				
Basal	11.36 ± 0.52	10.75 ± 0.46	-0.61 ± 0.40	0.138
10 mU/m²/min	7.59 ± 0.37	6.51 ± 0.42	-1.08 ± 0.28	0.001
40 mU/m²/min	2.04 ± 0.26	1.63 ± 0.22	-0.41 ± 0.19	0.045
Δ10-basal	-3.77 ± 0.39	-4.24 ± 0.36	-0.47 ± 0.42	0.269
Δ40-basal	-9.32 ± 0.50	-9.12 ± 0.35	0.20 ± 0.47	0.671
NOGD (μmol/kg/min)				
Basal	9.87 ± 0.78	8.47 ± 0.60	-1.40 ± 0.48	0.007
10 mU/m²/min	5.81 ± 0.47	4.90 ± 0.70	-0.91 ± 0.62	0.153
40 mU/m²/min	13.56 ± 1.35	15.55 ± 1.53	1.99 ± 1.04	0.066
Δ10-basal	-4.05 ± 0.54	-3.56 ± 0.72	0.49 ± 0.72	0.505
Δ40-basal	3.69 ± 1.56	7.08 ± 1.60	3.39 ± 1.08	0.004
CHO oxidation (μmol/kg/min)				
Basal	1.86 ± 0.60	2.49 ± 0.47	0.63 ± 0.50	0.217
10 mU/m²/min	4.69 ± 0.43	6.11 ± 0.60	1.42 ± 0.56	0.018
40 mU/m²/min	8.69 ± 0.61	9.24 ± 0.71	0.55 ± 0.51	0.294
Δ10-basal	2.83 ± 0.43	3.62 ± 0.49	0.79 ± 0.31	0.017
Δ40-basal	6.83 ± 0.65	6.75 ± 0.58	-0.08 ± 0.49	0.866
Lipid oxidation (μmol/kg/min)				
Basal	1.26 ± 0.05	1.21 ± 0.04	-0.05 ± 0.04	0.182
10 mU/m²/min	1.00 ± 0.04	0.86 ± 0.05	-0.13 ± 0.04	0.004
40 mU/m²/min	0.72 ± 0.04	0.64 ± 0.05	-0.08 ± 0.04	0.063
Δ10-basal	-0.27 ± 0.04	-0.35 ± 0.04	-0.08 ± 0.03	0.011
Δ40-basal	-0.55 ± 0.05	-0.57 ± 0.04	-0.02 ± 0.04	0.618
RER (arbitrary units AU)				
Basal	0.746 ± 0.009	0.755 ± 0.006	0.008 ± 0.007	0.228
10 mU/m²/min	0.787 ± 0.006	0.810 ± 0.008	0.022 ± 0.008	0.011
40 mU/m²/min	0.841 ± 0.007	0.852 ± 0.008	0.011 ± 0.007	0.106
Δ10-basal	0.041 ± 0.006	0.055 ± 0.008	0.014± 0.005	0.007
Δ40-basal	0.095 ± 0.009	0.097 ± 0.008	0.003 ± 0.007	0.710

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

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

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

DISCUSSION AND CONCLUSION

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

Free carnitine availability was previously reported to be reduced in T2DM patients (35). We here show that three months of carnitine supplementation elevates plasma free carnitine concentration. In addition, we investigated if skeletal muscle acetylcarnitine also increased. Interesting, a notable increase in resting skeletal muscle acetylcarnitine concentration as well as the capacity to form acetylcarnitine with exercise was observed after carnitine supplementation in type 2 diabetes patients, indicating that the supplemented carnitine reaches skeletal muscle and increases carnitine availability. These results are in line with our previous research in pre-diabetes individuals (9) where acetylcarnitine in muscle was increased after an even shorter period of supplementation. Together, these data support the notion that the capacity to form acetylcarnitine may be limited by availability of free carnitine (4, 6) and can be restored by supplementation.

We postulated that increasing free carnitine availability enhances the capacity to form acetylcarnitine, which in turn improves insulin sensitivity. In line with our hypothesis, three months of carnitine supplementation profoundly increased peripheral insulin sensitivity by 31% in type 2 diabetes patients. Similar results on markers of insulin sensitivity (HOMA-IR) were reported previously (10-12, 14, 36). However, here we used the gold standard hyperinsulinemic euglycemic clamp and show that the improvement in insulin sensitivity in the current study is mainly explained by insulin stimulated NOGD rather than glucose oxidation. Similar improvements in NOGD were previously reported after carnitine infusion during a hyperinsulinemic-euglycemic clamp in T2DM patients (37, 38). Glycogen synthesis in skeletal muscle tissue is known to be impaired in type 2 diabetes patients caused by a reduced stimulating effect of insulin on glycogen synthase (39). These observations suggest that carnitine may stimulate insulin-stimulated

glycogen synthesis, which is also supported by some in vitro results in the liver (40), however data on skeletal muscle is yet lacking.

Besides peripheral insulin sensitivity, we investigated for the first time the effect of three months of carnitine supplementation on hepatic insulin sensitivity in T2DM patients. Interestingly, hepatic insulin sensitivity improved by 22% after carnitine supplementation. Since hepatic insulin resistance is associated with increased ectopic lipid deposition in liver (41), we investigated whether a reduced IHL content is underlying the improvements in hepatic insulin sensitivity after carnitine supplementation. Indeed, a tendency towards lower IHL was found in the type 2 diabetes patients supplemented with carnitine. Next to potentially increasing fat oxidation in the liver (42), free carnitine can scavenge acyl-chains in the liver and export these as acylcarnitines to the blood. Studies investigating trans-organ acylcarnitine fluxes revealed that plasma acylcarnitines mainly reflect hepatic acylcarnitine export (43, 44). In line, we here reported elevated plasma concentrations of acetylcarnitine, medium- and long-chain acylcarnitines.

In line with the results on insulin sensitivity, we reported a tendency towards lower fasting plasma glucose levels in type 2 diabetes patients after carnitine supplementation. These lower fasting plasma glucose levels are consistent with Rahbar et al. (13), who also reported lower fasting plasma glucose levels after carnitine supplementation. The lack of statistical significance in the current study could be explained by the supplementation duration, since Asadi et al. (2020) recently revealed that minimally 12-weeks of carnitine supplementation is needed to establish a reduction in fasting plasma glucose levels (36). In type 2 diabetes patients, enhanced gluconeogenesis rates contribute to elevated fasting plasma glucose levels (45). Previously, it was reported that carnitine supplementation reduces gluconeogenesis in animals (46) which may potentially explain the tendency for lower fasting plasma glucose found here. Therefore, adding carnitine supplementation to the conventional treatment of T2DM patients might be a good strategy to reduce hyperglycemia, highlighting the clinical relevance of carnitine supplementation. The current study did not show improvements in HbA_{1c} levels. Since the HbA_{1c} measurement comprises a 3-month average of glycated hemoglobin, it is likely that the supplementation duration of three months in the current study is too short to induce changes. Indeed, studies with similar or shorter supplementation duration failed to improve HbA_{1c} (13, 47, 48), whilst reductions in HbA_{1c} are reported with longer study durations (48 weeks) (11, 36, 49).

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

A limitation of our study is the absence of a placebo-controlled group. Study-induced changes in energy intake and physical activity are prone to placebo effects. Therefore, we monitored these factors but did not report any changes after three months of carnitine supplementation, implicating no contribution of life-style change to a possible placebo effect. Nevertheless, to estimate the magnitude of a possible placebo effect on insulin sensitivity, we determined the changes in the control groups in various interventional studies with similar designs and similar participant population (type 2 diabetes patients) (50-53). In these studies, a maximum placebo-induced effect of $+8\%$ on insulin sensitivity was reported in the placebo-controlled groups. In the current study, the reported effect of carnitine supplementation on insulin sensitivity is very pronounced reaching a 31% increase. Therefore, we are convinced that the pronounced effect on insulin sensitivity in the current study indeed originates from the administrated carnitine. The absence of a control group might be most problematic for the explorative investigated parameters reported in supplementary table 3 (such as 6MWT and TCST tests). However, it should be noted that when comparing the effect of carnitine to the effect of placebo supplementation as reported earlier, 50-100% stronger improvements were found in the current study (54). Therefore, we believe that carnitine supplementation improves daily life functioning, though longer and larger studies including a classic control are needed to confirm these results.

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

indicating patients also perceived benefits themselves from the applied intervention. However, future research including a classical placebo-controlled trial is necessary. In conclusion, we demonstrated that daily carnitine supplementation might be an interesting add-on therapy in patients with type 2 diabetes.

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Declaration of interest

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

Author contributions

Conceptualization, Y.M.H.B., R.B.B., A.V., M.K.C.H., P.S. and V.B.S.-H.; Methodology, Y.M.H.B., E.P., M.K.C.H., P.S. and V.B.S.-H.; Formal Analysis, Y.M.H.B., P.V., G.S. and E.K.; Investigation, Y.M.H.B., Y.J.M.K. and B.H.; Writing – Original Draft, Y.M.H.B.; Writing – Review & Editing, Y.J.M.K., P.V., E.P., B.H., G.S., R.B.B., A.V., J.E.W., M.K.C.H., P.S. and V.B.S.-H.; Supervision, V.B.S.-H., J.E.W., M.K.C.H. and P.S.; Project administration, Y.M.H.B., V.B.S and P.S.; Funding acquisition, V.B.S.-H.

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SUPPLEMENTARY

Table S1. Participant characteristics at screening

Variable	Type 2 diabetes patients (n=32)
Gender (m/f), n	21/11
Age (y)	65 ± 1
Body weight (kg)	89.4 ± 2.8
Body mass index (kg/m^2)	30.0 ± 1.0
Fat mass (kg)	33.6 ± 1.8
Fat free mass (kg)	55.8 ± 1.7
Fat percentage (%)	37.2 ± 1.4
Systolic blood pressure (mmHg)	142 ± 2.0
Diastolic blood pressure (mmHg)	87 ± 1.3
Fasting glucose (mmol/L)	8.2 ± 0.3
Fasting insulin (pmol/L)	84.6 ± 8.5
HbA_{1c} (mmol/mol)	48.9 ± 1.5
Triglycerides (mmol/L)	1.6 ± 0.1
Total cholesterol (mmol/L)	4.4 ± 0.2
HDL cholesterol (mmol/L)	1.3 ± 0.1
LDL cholesterol (mmol/L)	2.4 ± 0.2
VO_{2max} (ml/min/kg)	24.4 ± 0.9
W_{max} (W/kg)	1.61 ± 0.1

Data are presented as mean ± SEM. HbA_{1c}, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low density lipoprotein; W_{max}, maximal workload

Table S2. Plasma acylcarnitine profiles of the type 2 diabetes patients at baseline and 3 months of carnitine supplementation

Variable (mmol/kgww)	Baseline	Minimal-maximum	Month 3	Minimal-maximum	Delta [a]	P-value
C0	37.95 ± 1.47	14.74-52.01	59.35 ± 2.00	39.57-91.14	21.40 ± 1.63	0.000
C2	6.41 ± 0.39	3.05-11.84	11.16 ± 0.54	4.89-17.57	4.75 ± 0.40	0.000
C3	0.44 ± 0.02	0.11-0.79	0.74 ± 0.04	0.39-1.35	0.30 ± 0.03	0.000
C4	0.26 ± 0.02	0.07-0.63	0.39 ± 0.03	0.17-1.01	0.13 ± 0.02	0.000 [b]
C5:1	0.01 ± 0.00	0.00-0.02	0.01 ± 0.00	0.00-0.02	0.00 ± 0.00	0.057
C5	0.13 ± 0.01	0.05-0.22	0.16 ± 0.01	0.07-0.34	0.03 ± 0.01	0.001
C4-3OH	0.04 ± 0.00	0.02-0.08	0.08 ± 0.01	0.02-0.17	0.05 ± 0.01	0.000
C6	0.05 ± 0.00	0.03-0.13	0.08 ± 0.01	0.04-0.32	0.03 ± 0.01	0.000 [b]
C5OH	0.02 ± 0.00	0.00-0.04	0.02 ± 0.00	0.00-0.04	0.01 ± 0.00	0.002 [b]
C8	0.17 ± 0.01	0.07-0.36	0.22 ± 0.02	0.10-0.59	0.05 ± 0.01	0.000 [b]
C3DC	0.04 ± 0.00	0.02-0.11	0.05 ± 0.00	0.02-0.11	0.01 ± 0.00	0.000 [b]
C10:1	0.12 ± 0.01	0.03-0.26	0.15 ± 0.01	0.07-0.28	0.03 ± 0.01	0.000 [b]
C10	0.23 ± 0.02	0.07-0.57	0.29 ± 0.03	0.10-0.79	0.06 ± 0.02	0.003 [b]
C4DC	0.04 ± 0.00	0.03-0.06	0.04 ± 0.00	0.03-0.07	0.00 ± 0.00	0.029 [b]
C5DC	0.04 ± 0.00	0.01-0.09	0.04 ± 0.00	0.02-0.09	0.01 ± 0.00	0.000 [b]
C12:1	0.07 ± 0.01	0.02-0.19	0.09 ± 0.01	0.04-0.16	0.02 ± 0.00	0.001 [b]
C12	0.06 ± 0.00	0.02-0.14	0.07 ± 0.01	0.03-0.14	0.02 ± 0.00	0.001 [b]
C6DC	0.02 ± 0.00	0.01-0.04	0.02 ± 0.00	0.01-0.04	0.00 ± 0.00	0.366 [b]
C12:1OH	0.02 ± 0.00	0.01-0.04	0.02 ± 0.00	0.01-0.05	0.01 ± 0.00	0.001 [b]
C12OH	0.01 ± 0.00	0.00-0.02	0.01 ± 0.00	0.00-0.02	0.00 ± 0.00	0.025 [b]
C14:2	0.04 ± 0.00	0.01-0.13	0.06 ± 0.00	0.03-0.11	0.01 ± 0.00	0.001 [b]
C14:1	0.11 ± 0.01	0.04-0.32	0.14 ± 0.01	0.07-0.27	0.03 ± 0.01	0.000 [b]
C14	0.04 ± 0.00	0.02-0.07	0.05 ± 0.00	0.03-0.07	0.01 ± 0.00	0.000 [b]
C8DC	0.01 ± 0.00	0.00-0.01	0.01 ± 0.00	0.00-0.04	0.00 ± 0.00	0.001 [b]
C14:1OH	0.02 ± 0.00	0.01-0.03	0.02 ± 0.00	0.01-0.04	0.01 ± 0.00	0.001 [b]
C14OH	0.01 ± 0.00	0.00-0.02	0.02 ± 0.00	0.00-0.03	0.01 ± 0.00	0.000 [b]
C16:1	0.03 ± 0.00	0.01-0.06	0.04 ± 0.00	0.02-0.06	0.01 ± 0.00	0.000 [b]
C16	0.11 ± 0.00	0.07-0.17	0.13 ± 0.01	0.09-0.21	0.02 ± 0.00	0.000 [b]
C10DC	0.01 ± 0.00	0.00-0.02	0.02 ± 0.00	0.00-0.02	0.01 ± 0.00	0.000 [b]
C16:1OH	0.01 ± 0.00	0.00-0.01	0.01 ± 0.00	0.00-0.01	0.00 ± 0.00	0.059 [b]
C16OH	0.00 ± 0.00	0.00-0.01	0.01 ± 0.00	0.00-0.02	0.01 ± 0.00	0.000 [b]

C18:2	0.03 ± 0.00	0.02-0.08	0.04 ± 0.00	0.02-0.06	0.01 ± 0.00	0.002 [b]
C18:1	0.11 ± 0.01	0.05-0.21	0.13 ± 0.01	0.08-0.20	0.02 ± 0.00	0.000 [b]
C18	0.04 ± 0.00	0.02-0.05	0.04 ± 0.00	0.03-0.07	0.01 ± 0.00	0.001 [b]
C18:2OH	0.00 ± 0.00	0.00-0.00	0.00 ± 0.00	0.00-0.00	0.00 ± 0.00	1.000 [b]
C18:1OH	0.00 ± 0.00	0.00-0.01	0.01 ± 0.00	0.00-0.01	0.00 ± 0.00	0.000 [b]
C18OH	0.00 ± 0.00	0.00-0.01	0.01 ± 0.00	0.00-0.02	0.01 ± 0.00	0.000 [b]
Short	1.04 ± 0.04	0.34-1.63	1.55 ± 0.07	1.00-2.76	0.53 ± 0.05	0.000
Medium	0.78 ± 0.06	0.29-1.48	0.98 ± 0.07	0.53-2.21	0.22 ± 0.05	0.000
Long	0.54 ± 0.03	0.26-1.13	0.68 ± 0.03	0.39-1.03	0.15 ± 0.02	0.000

Data are presented as mean ± SEM. [a] delta: month 6-baseline values. [b] data are not normally distributed and non-parametrically tested.

Table S3. General daily functioning (i.e. physical performance, QoL, QoS and cognitive performance) at baseline and after 3 months of carnitine supplementation

Variable	Baseline	Month 3	Delta [a]	P-value
Physical performance				
6MWT (m)	459.04 ± 11.44	478.97 ± 12.30	19.93 ± 4.59	0.000
TCST (s)	25.01 ± 0.93	22.66 ± 0.77	-2.36 ± 0.63	0.000
Quality assessment				
Quality of Sleep	6.77 ± 0.43	6.68 ± 0.49	-0.09 ± 0.26	0.741 [b]
Quality of Life	121.19 ± 3.60	122.13 ± 3.48	1.03 ± 2.00	0.609
Cognitive performance				
MOT LM (ms)	892 ± 34	848 ± 41	-44 ± 19	0.042 [b]
RTI MT (ms)	324 ± 12	328 ± 14	4 ± 9	0.510 [b]
RTI RT (ms)	417 ± 9	424 ± 10	7 ± 5	0.381 [b]
MTT IC (ms)	115 ± 12	118 ± 12	3 ± 10	0.818
MTT MTC (ms)	364 ± 32	315 ± 22	-49 ± 26	0.097
MTT ML (ms)	795 ± 18	814 ± 19	19 ± 15	0.284 [b]
MTT IN	10 ± 2	4 ± 1	-6 ± 2	0.002 [b]
SSP FSL	5 ± 0.2	6 ± 0.2	0.3 ± 0.2	0.093 [b]
DMS TC (%)	82 ± 2	83 ± 2	1 ± 2	0.626
PAL FAMS	11 ± 1	12 ± 1	2 ± 1	0.024
PAL TE	15 ± 1	13 ± 1	-2 ± 1	0.104

Data are presented as mean ± SEM. [a] delta: month 6-baseline values; [b] data are not normally distributed and non-parametrically tested. 6MWT, 6-minute walk test; TCST, Timed Chair-Stand Test; Cognitive performance was evaluated using the domains psychomotor speed (MOT, RTI), executive function (MTT, SSP) and memory (DMS, PAL). MOT, motor screening task; RTI, reaction time; MTT, multitasking test; SSP, spatial span; DMS, delayed matching to sample; PAL, paired associates learning; LM, mean latency; MT, movement time; RT, reaction time; IC, incongruency cost; MTC, multitasking cost; ML, median latency; IC, total incorrect (or total errors); FSL, forward span length; TC, total correct; FAMS, first attempt memory score; TE, total errors. Physical performance represents n=31, one participant was unable to perform these test due to an injury on the foot. Cognitive performance represents n=29, three participants failed to perform the cognitive performance test due to a technical error.

Chapter 6

Effects of the SGLT2 inhibitor dapagliflozin on energy metabolism in patients with type 2 diabetes: a randomized, double-blind crossover trial

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ABSTRACT

Background: SGLT2 inhibitors increase urinary glucose excretion and have beneficial effects on cardiovascular and renal outcomes; the underlying mechanism may involve caloric restriction-like metabolic effects due to urinary glucose loss. We investigated the effects of dapagliflozin on 24h energy metabolism and insulin sensitivity in patients with type 2 diabetes mellitus.

Methods: Twenty-six type 2 diabetes patients were randomized to a 5-week double-blind, cross-over study with 6-8-week wash-out. 24h energy metabolism and respiratory exchange ratio (RER) were measured by indirect calorimetry, both by whole-room calorimetry and by ventilated hood during a two-step euglycemic hyperinsulinemic clamp. Results are presented as the differences in least squares mean (LSM) (95% CI) between treatments.

Results: Evaluable patients (n=24) had a mean (SD) age of 64.2(4.6) years, BMI of 28.1(2.4) kg/m^2, and HbA_{1c} of 6.9(0.7)% (51.7 (6.8) mmol/mol). Rate of glucose disappearance was unaffected by dapagliflozin, while fasting endogenous glucose production (EGP) increased by dapagliflozin (+2.27 (1.39, 3.14) μmol/kg/min, p<0.0001). Insulin-induced suppression of EGP (-1.71 (-2.75, -0.63) μmol/kg/min, p=0.0036) and plasma free fatty acids (-21.93 (-39.31, -4.54) %, p=0.016) was greater with dapagliflozin. 24h energy expenditure (-0.11 (-0.24, 0.03) MJ/day) remained unaffected by dapagliflozin, but dapagliflozin reduced RER during day- and night-time resulting in an increased day to night-time difference in RER (-0.010 (-0.017, -0.002), p=0.016). Dapagliflozin treatment resulted in a negative 24h energy and fat balance (-20.51 (-27.90, -13.12) g/day).

Conclusions: Dapagliflozin treatment for 5 weeks resulted in major adjustments of metabolism mimicking caloric restriction; increased fat oxidation, improved hepatic and adipose insulin sensitivity and improved 24h energy metabolism.

INTRODUCTION

Sodium-glucose cotransporter 2 inhibitors (SGLT2i) inhibit glucose and sodium reabsorption in the proximal renal tubules and have been proven a valuable additional treatment for type 2 diabetes to improve glycemic control and also moderately reduce body weight and blood pressure (1). Importantly, SGLT2i have been shown to reduce cardiovascular (CV) risk, including reduced hospitalization for heart failure and reduced risk of kidney disease progression (2, 3). How SGLT2i exerts its remarkable effects on CV and renal outcomes is not fully understood. For example, only 12% of the relative risk reduction for all-cause death caused by SGLT2i could be explained by the combined changes in conventional cardiovascular risk factors (4).

The primary action of SGLT2i on glucose and sodium reabsorption has several metabolic consequences that may help to explain the effects on CV and renal outcome (1). Such metabolic effects may reflect the adaptive response to the loss of about 50-100g glucose per day in the urine, resulting in a form of mild caloric restriction and caloric loss overnight. Consistent with this line of thinking, SGLT2i treatment has been reported to result in increased glucagon levels (5), and decreased insulin secretion (6), most likely mediated by decreased plasma glucose levels (7). Other observed metabolic effects include increased endogenous glucose production (EGP) (8), increased fasting fatty acid oxidation (9), and decreased intrahepatic lipid (IHL) content (10). In addition, several (8, 9, 11) but not all (10) studies have reported improvements in whole-body/peripheral insulin sensitivity. All these separate effects resemble effects that are also observed after caloric restriction, which is also accompanied by enhanced fat oxidation (12), improved metabolic flexibility (13), reductions in plasma glucose and improvements in insulin sensitivity (13). Despite these separate finding, so far no studies have investigated the hypothesis that SGLT2i treatment of type 2 diabetes patients can indeed restore the fed to fasting cycle in type 2 diabetes patients and if the treatment has caloric-restriction like effect on 24h energy and substrate metabolism, as was hypothesized previously (14).

Therefore, the aim of the current study was to investigate if SGLT2i treatment for 5 weeks affects 24h energy balance and substrate metabolism in type 2 diabetes patients. To this end, whole-body 24h energy expenditure and substrate oxidation, whole-body and tissue-specific insulin sensitivity and body composition including ectopic fat accumulation were investigated in large detail in type 2 diabetes patients after 5 weeks of SGLT2i or placebo treatment.

METHODS

Study design and participants

A double-blind, randomized, placebo-controlled, cross-over study was conducted at the Metabolic Research Unit Maastricht (MRUM) of Maastricht University between March 5 2018 and November 4 2019. Ethics Committee of Maastricht University Medical Centre approved the study, which was conducted conform to Helsinki declaration (15). Upon written informed consent, patients were randomized to double-blind treatment for two treatment periods, each 5 weeks or maximum duration of 40 days, separated by a 6-8 weeks wash-out (see supplementary for details). Endpoints were assessed at the end of each 5-week period. Male and female patients with type 2 diabetes, diagnosed for at least 6 months, with HbA_{1c} levels between 6 and 9 % (42 and 75 mmol/mol), and on a stable dose of metformin for >3 months or drug naïve, were eligible. Detailed eligibility criteria are in Table S1.

Procedures

The study comprised seven visits, including screening (Visit 1). Visit 2 (or 5), the first visit of the treatment periods, included randomisation and measurement of safety markers. A safety visit (Visit 3 or 6) was scheduled after two weeks, and an end-of-treatment visit (Visit 4 or 7) after 5 weeks. All outcome measures were performed at Visit 4/7 and were spread over a period of 6-8 days and visit 4 was followed by 6-8 weeks wash-out period. Timing of study medication during end-of treatment was around 08:30 A.M., except on the day of the euglycemic hyperinsulinemic clamp (EHC), when the timing of medication was at 06:30 A.M., before start of tracer infusion.

During the end-of-treatment visit, blood was drawn at 01:30 PM for assessment of HbA_{1c}, uric acid, and high-sensitivity C-reactive protein. Hereafter, participants stayed in a respiration chamber from 06:00 PM onwards to measure O_2 consumption and CO_2 production (16). The last 24h starting at 05:30 AM were used for analysis. In the respiration chamber, participants adhered to an activity protocol consisting of multiple times standing upright and including twice a 15-minute stepping exercise at 30 steps/min to increase physical activity levels. Physical activity was measured by Actigraphy (ActigraphCorp, Florida, USA). Participants received three standardized meals at 08:30, 13:00 and at 18:00. 24h energy intake was determined from sleeping metabolic rate (SMR) during the first night multiplied by an activity factor of 1.5, and

all meals were completely consumed by the volunteers. Twenty-four hour energy expenditure and respiratory exchange ratio was calculated using Brouwer et al (17). Daytime RER was calculated from 08:00 till 22:00 and night-time RER from 00:00 till 05:30. SMR was defined as the two consecutive hours with lowest energy expenditure using Weir formula (18), and was averaged for 2 nights. 24h diet induced thermogenesis was determined by plotting energy expenditure against physical activity level, and intercept of the regression-line at lowest physical activity represents SMR+DIT (19). 24-hour urine collection (in 6-hour aliquots) were collected for measurement of glucose and nitrogen, the latter to calculate daytime and night-time protein oxidation. Blood samples were drawn at seven timepoints (see Table S2).

After leaving the respiration chamber after an overnight fast, proton magnetic resonance spectroscopy (MRS) was used to quantify IHL content (see supplementary for details). Subsequently, a EHC with co-infusion of D-glucose D-[6,6-$^2H_2$] tracer (0.04 mg/kg/min) was performed (20). After 3 hours, an insulin infusion was started with 10mU/m^2/min for 3h to assess hepatic insulin sensitivity and subsequently increased to 40mU/m^2/min for 2.5h to assess whole-body insulin sensitivity. 20% glucose was co-infused to keep glucose levels at ~5 mmol/L. Arterialized blood (hotbox) was drawn every 5-10 minutes, to monitor glucose concentration. During the last 30 minutes of every steady state period, blood samples were collected and indirect calorimetry (Omnical, Maastricht Instruments, Maastricht) was performed to assess substrate utilization according to Péronnet et al (21). Non-oxidative glucose disposal (NOGD) was defined as rate of disappearance (R_d) corrected for urinary glucose loss minus carbohydrate oxidation. Metabolic flexibility was defined as the change in respiratory exchange ratio from basal to high-insulin stimulated state. Isotopic enrichment of plasma glucose was determined using electron ionization gas chromatography-mass spectrometry (22). Calculation of glucose rate of appearance (R_a) and R_d was performed according to Steele's single pool non-steady state equations (23). Basal R_d values were corrected for fasting urinary glucose excretion during the night, and Rd values during insulin infusion were corrected for urinary glucose excretion during the clamp. In two participants, glucose excretion during the clamp could not be measured in one of the periods, and fasted urinary glucose excretion was used, multiplied by the percent average difference on urinary glucose excretion in the fasted (2.209 and 0.008 μmol/kg/min on dapagliflozin and placebo) and insulin-stimulated state (2.656 and 0.006 μmol/kg/min on dapagliflozin and placebo). Exclusion of these two subjects did not alter the EHC results.

On day 6, 7 or 8 during the end-of-treatment visit, a Dual Energy X-ray Absorptiometry (DEXA) scan (Hologic, Inc., MA, USA) was used to determine body composition.
Changes in vital sign and safety lab values were followed during the study. Adverse events leading to discontinuation from the study as well as serious adverse events and cases of suspected diabetic ketoacidosis were collected.

Biochemical analysis
Details of biochemical analysis are outlined in Supplementary materials.

Statistics
The evaluable analysis set, consisting of patients with at least one dose of the investigational product (per protocol) and no important protocol deviations, was used for the statistical analyses, using SAS® software version 9.04 and performed by IQVIA Biostatistics, Reading, United Kingdom. The expected difference between treatment groups was estimated using a linear mixed effects model. This model had treatment group, treatment sequence and period as fixed effects, as well as random intercept for each subject. If deviations from normality was detected, a non-parametric test of treatment difference against zero was performed (Wilcoxon signed-rank test) using all the data and ignoring the sequence. The least-squares means (LSM) and the corresponding 95% CIs for treatment effect in the respective treatment periods, are presented. The difference in LSM between the two treatments was computed, with corresponding 95% CI and p-value tabulated. A two-sided 0.05 level was considered as statistically significant. We used the Benjamini-Hochberg procedure for multiple testing and found that adjusted p-values were in agreement with unadjusted p-values. For power calculation, see supplementary materials.

The trial is registered with ClinicalTrials.gov, number NCT03338855.

RESULTS

Thirty-eight patients were enrolled, and 26 patients were randomized (Figure S1). Twelve patients were ineligible for participation. Two patients were excluded from the evaluable data set because of important protocol deviations. One of the excluded participants discontinued the study after completing one period as a result of change in medical treatment affecting insulin sensitivity. The other patient was excluded because safety and efficacy assessments were not performed as defined in the protocol.

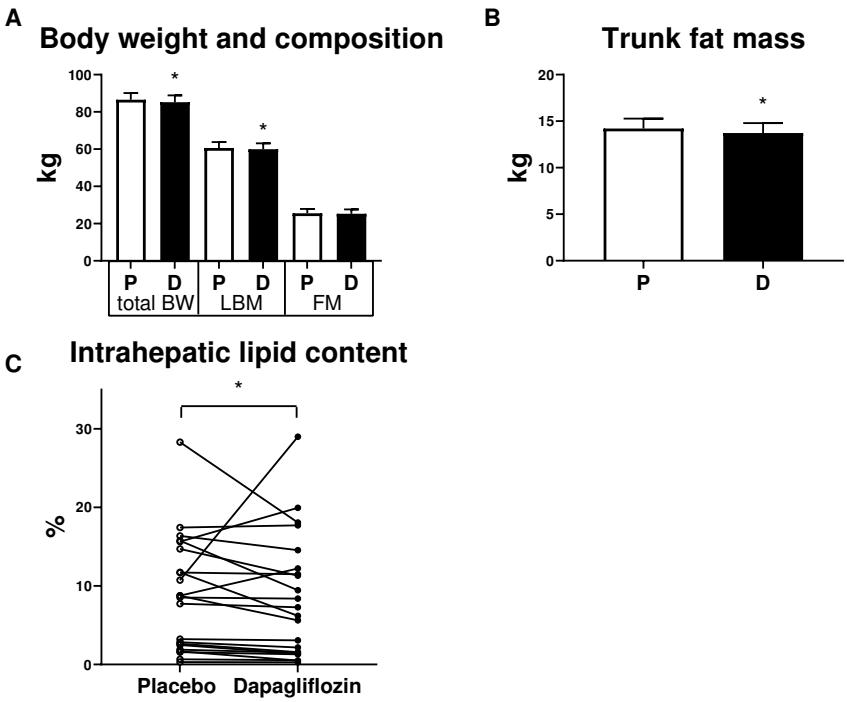

Figure 1. a) Body composition (n=24), b) fat mass (n=24) and c) intrahepatic lipid (IHL) content upon placebo (P) and dapagliflozin (D) treatment (n=22). BD = body weight, LBM = lean body mass, FM = fat mass. ○ = placebo and ● = dapagliflozin. Results are in least squares mean (LSM) and 95% confidence interval (CI), obtained through a linear mixed model, with exception of IHL, where a Wilcoxon paired rank sum test is used. * P<0.05 is considered significantly different.

Baseline characteristics of the 24 evaluable patients are reported in Table S3. The patients had a mean (SD) age of 64.2 (4.6) years, BMI of 28.1 (2.4) kg/m^2 and HbA_{1c} of 6.9 (0.7) % / 51.7 (6.8) mmol/mol. Patients had a mean eGFR of 141 (13.0)

ml/min. Seventeen patients were on metformin treatment and seven patients did not use any antidiabetic drugs. No other anti-diabetic drugs were used. Compliance was >95% during both treatment periods. Body weight was significantly reduced by dapagliflozin treatment compared to placebo (-1.26 (-1.85, -0.66) kg, p=0.0003, Figure 1a). Body weight was measured also at the beginning of each period, and no carry-over effects as a result of treatment sequence were noticed. Assessment of body composition by DEXA showed significantly reduced lean mass by dapagliflozin treatment compared to placebo (-0.67 (-1.29, -0.04) kg, p=0.038, Figure 1a), whereas whole-body fat mass was not significantly affected by dapagliflozin treatment (Figure 1a). Trunk fat mass (-0.48 (-0.89, -0.07) kg, p=0.023, Figure 1b) as well as IHL content, as measured by ^{1}H-MRS, were lower after dapagliflozin treatment (18 out of 22 patients, p=0.036, Figure 1c).

Systolic blood pressure after two weeks of treatment was significantly lower after dapagliflozin treatment (-6.77 (-12.09, -1.45) mmHg, p=0.015, table S4), but diastolic blood pressure was not significantly affected by dapagliflozin (-1.85 (-5.68, 1.98) mmHg, p=0.33, Table S4). Systolic blood pressure (-3.58 (-10.33, 3.16) mmHg, p=0.28, Table S4) or diastolic blood pressure (-0.65 (-4.61, 3.31) mmHg, p=0.74, Table S4,) after 5 weeks of treatment was not significantly changed after dapagliflozin treatment. Levels of plasma hsCRP (0.22 (-0.45, 0.90) mg/L, p=0.50, table S5,) and HbA_{1c} (-0.07 (-0.22, 0.08) %, p=0.33, Table S5), measured during the end-of-treatment visits, were not significantly altered with dapagliflozin treatment. HbA_{1c} was measured also at the beginning of each period, and no carry-over effects as a result of treatment sequence were noticed with respect to HbA_{1c} levels. Urate levels were lower after dapagliflozin treatment (-56.8 (-75.4, -38.1) μmol/L, p<0.0001, table S5). Furthermore, levels of hemoglobin were higher with dapagliflozin treatment (0.19 (0.02, 0.35) mmol/L, p=0.03, Table S5), while there was no significant change in erythrocyte volume fraction (0.004 (-0.006, 0.014) L/L, p=0.43, Table S5).

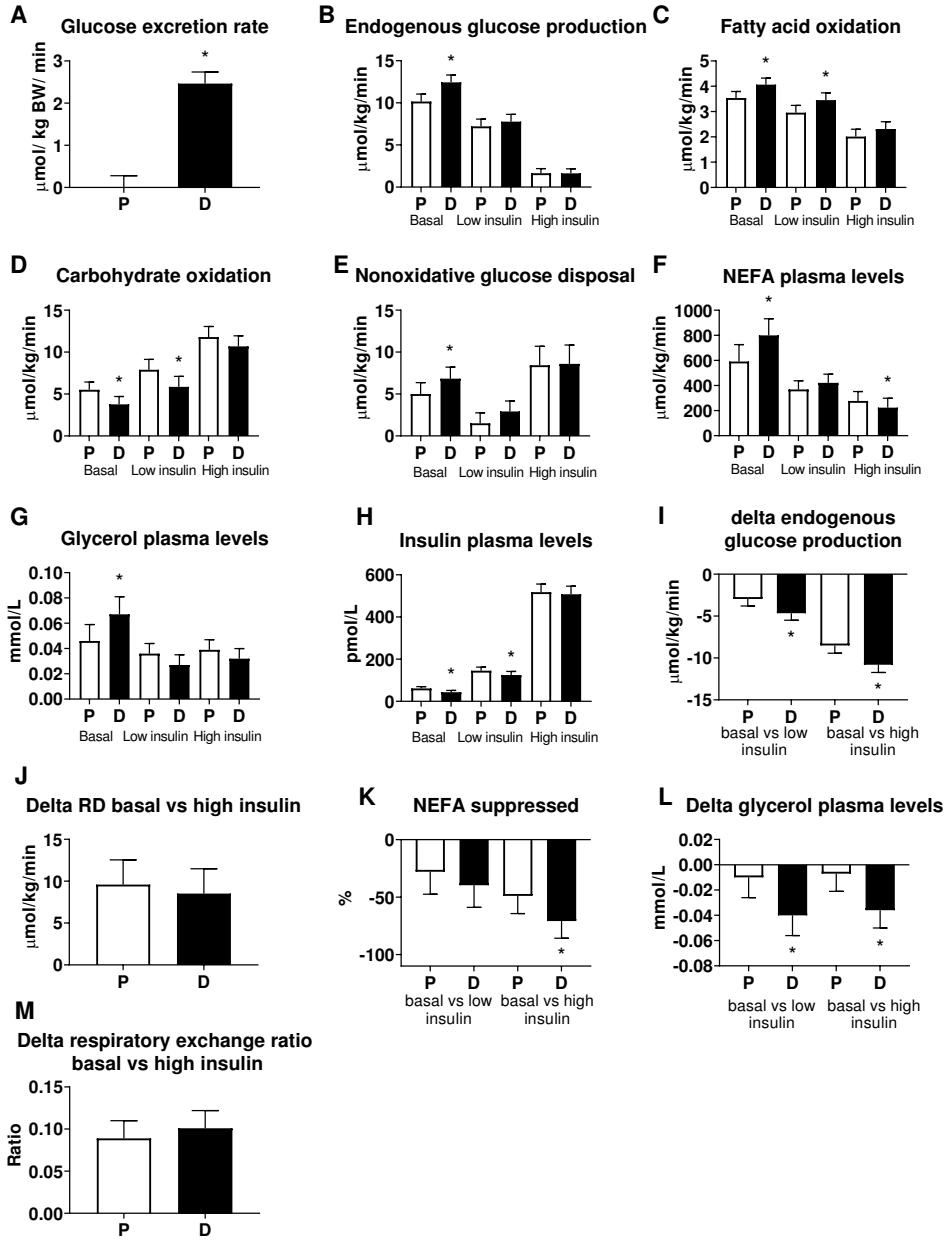

Figure 2. a) urinary glucose excretion, b) endogenous glucose production (EGP), c) fatty acid (FA) oxidation, d) carbohydrate (CHO) oxidation, e) nonoxidative glucose disposal (NOGD), f) plasma non-esterified fatty acid (NEFA) levels, g) plasma glycerol levels, h) plasma insulin levels, i) delta EGP, j) delta R_d (rate of disposal), k) %NEFA suppression, l) delta suppression of glycerol plasma levels and m) delta respiratory exchange ratio (RER) measured during a euglycemic hyperinsulinemic two-step clamp upon placebo (P) and dapagliflozin (D) treatment. Results (n=22) are in least squares mean (LSM) and 95% confidence interval (CI) , obtained through a linear mixed model. * $P<0.05$ is considered significantly different.

A two-step EHC with indirect calorimetry was performed after an overnight fast when the patients had been in the respiration chamber for 36h. Urinary glucose excretion during the clamp increased after dapagliflozin treatment (2.46 (2.06, 2.86) μmol/kg/min, p<0.0001, Table S6, Figure 2a). Basal or fasting EGP was higher after dapagliflozin treatment (2.27 (1.39, 3.14) μmol/kg/min, p<0.0001, Table S6, Figure 2b); an increase that was similar in magnitude to the increase in urinary glucose excretion rate. Basal R_d, corrected for urinary glucose loss, was similar between the dapagliflozin and placebo treatment periods (0.11 (-1.12, 1.25) μmol/kg/min, p=0.85, Table S6). Fasting fat oxidation was higher (0.53 (0.22, 0.85) μmol/kg/min, p=0.0022, Table S6, Figure 2c), while fasting carbohydrate oxidation was lower after dapagliflozin treatment as compared to placebo (-1.73 (-2.72, -0.74), μmol/kg/min, p=0.0016, Table S6, Figure 2d). NOGD in the fasted state was higher with dapagliflozin treatment versus placebo (1.85 (0.45, 3.24) μmol/kg/min, p=0.012, Table S6, Figure 2e). Fasting NEFA levels (208.88 (53.88, 363.89) μmol /L, p=0.011, Table S6, Figure 2f) and fasting glycerol levels (0.022 (0.005, 0.039) mmol/L, p=0.013, Table S6, Figure 2g) were all significantly higher after dapagliflozin treatment compared to placebo. Fasting insulin levels were lower after dapagliflozin treatment as compared to placebo (-18.18 (-23.07, -13.28) pmol/L, p<0.0001, Table S6, Figure 2h).

Next, a low dose of insulin (10mU/m²/min) was infused for 3h to assess hepatic insulin sensitivity. The insulin-induced suppression (ΔEGPlow-basal) was larger with dapagliflozin treatment as compared to placebo (-1.71 (-2.78, -0.63) μmol/kg/min, p=0.0036, Table S6, Figure 2i) suggestive of enhanced hepatic insulin sensitivity. Improvements in insulin suppression of EGP were observed despite lower plasma insulin levels after dapagliflozin treatment (-20.73 (-37.62, -3.83) pmol/L, p=0.019, Table S6, Figure 2h). In the low-insulin state, fat oxidation was significantly higher (0.50 (0.11, 0.89) μmol/kg/min, p=0.015, Table S6, Figure 2c), whereas carbohydrate oxidation was significantly lower after dapagliflozin treatment as compared to placebo (-2.03 (-3.85, -0.21) μmol/kg/min, p=0.030, Table S6, Figure 2d).

In order to assess peripheral insulin sensitivity, a high dose (40mU/m²/min) of insulin was subsequently infused for 2.5h. Insulin sensitivity expressed as the change in R_d from basal to the high-insulin state and corrected for urinary glucose loss ($\Delta R_{d\ high-basal}$), was not significantly affected by dapagliflozin (-1.07 (-3.18, 1.05) μmol/kg/min, p=0.33, Table S6). Plasma insulin levels during high-insulin infusion were not affected

by dapagliflozin (-9.73 (-36.54, 17.09) pmol/L, p=0.46, Table S6, Figure 2h), and $\Delta R_{d\ high-basal}$ corrected for steady state insulin concentrations was not different between the treatment arms (-0.0029 (-0.0083, 0.0024), p=0.27, Table S6, Figure 2j). Plasma NEFA was more suppressed upon high insulin infusion after dapagliflozin treatment as compared to placebo (-21.93 (-39.31, -4.54) %, p=0.016, Table S6, Figure 2k), resulting in lower NEFA levels during the high-insulin state after dapagliflozin treatment versus placebo (-53.21 (-101.2, -5.23) µmol/L, p=0.031, Table S6, Figure 2f). Similarly, plasma glycerol levels were more suppressed by high insulin infusion after dapagliflozin treatment compared to placebo (-0.029 (-0.050, -0.008) mmol/L, p=0.0085, Table S6, Figure 2l). Differences in fatty acid oxidation (0.30 (-0.01, 0.60) µmol/kg/min, p=0.055, Table S6, Figure 2c) or carbohydrate oxidation (-1.11 (-2.33, 0.11) µmol/kg/min, p=0.071, Table S6, Figure 2d) between dapagliflozin and placebo during the high-insulin state did not reach statistical significance. NOGD during high insulin infusion rate was not changed by dapagliflozin (0.16 (-1.89, 2.22) µmol/kg/min, p=0.87, Table S6, Figure 2e). The insulin-induced increase in carbohydrate oxidation and reduction in fatty acid oxidation were more pronounced after dapagliflozin treatment but did not reach statistical significance (ΔCHOox $_{high-basal}$, 0.77 (-0.37, 1.92) µmol/kg/min, p=0.17, Table S6), (ΔFAox $_{high-basal}$, -0.28 (-0.65, 0.09) µmol/kg/min, p=0.13, Table S6). Changes in RER between basal and high-insulin state (as a measure or metabolic flexibility) were numerically larger after dapagliflozin treatment but did not reach statistical significance (0.01 (-0.01, 0.03), p=0.18, Table S6, Figure 2m).

To investigate the effect of dapagliflozin on whole-body substrate metabolism in detail, patients stayed in a respiration chamber for 36 hours and measurements were performed during the last 24h of the stay. Twenty-four-hour total energy expenditure was not significantly affected by dapagliflozin treatment compared to placebo (-0.11 (-0.25, 0.03) MJ/day, p=0.11, Table S7, Figure 3a). Sleeping metabolic rate was not significantly affected by dapagliflozin treatment (p=0.36, Table S7). Food intake was similar during both treatment periods and activity levels did not differ between the two periods (6.33 (-9.65, 22.32) counts/min, p=0.42). Diet induced thermogenesis was not significantly affected by dapagliflozin treatment (p=0.64, Table S7).

Twenty-four-hour urinary glucose loss was significantly higher by dapagliflozin treatment compared to placebo (3.53 (3.04, 4.00) g/h, p<0.0001, Table S7). Interestingly, urinary

glucose loss during dapagliflozin treatment was ~2-fold higher at daytime versus nighttime (4.3 (3.7, 5.0) g/h vs 2.0 (1.6, 2.4) g/h (Figure 3b and c). After correction for energy lost as urinary glucose, energy balance was negative in the dapagliflozin period (Figure 3d).

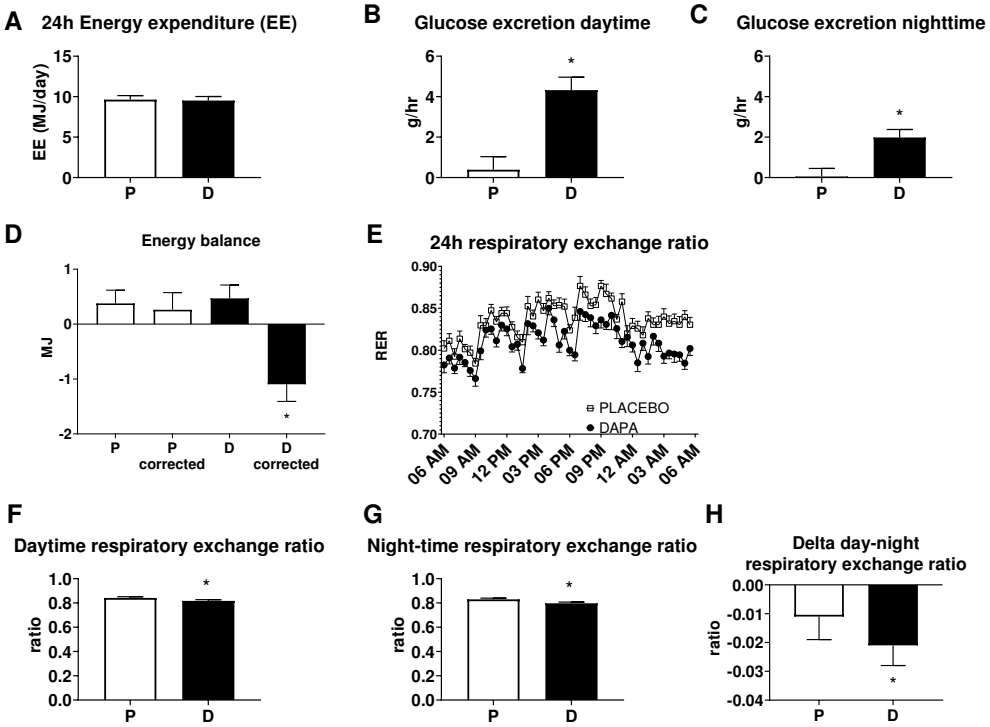

Figure 3. a) 24h energy expenditure (n=24), b) Glucose excretion daytime (n=23), c) glucose excretion nighttime (n=23), d) energy balance (n=24), e) 24h respiratory exchange ratio (RER) plot (n=24), f) daytime RER (n=24), g) nighttime RER (n=24), h) delta RER between day and night (n=24). Results are in least squares mean (LSM) and 95% confidence interval (CI) , obtained through a linear mixed model. * P<0.05 is considered significantly different

Twenty-four-hour RER was lower after dapagliflozin treatment (-0.02 (-0.03, -0.01), p=0.0001, Table S7, Figure 3e). This effect was observed both during daytime (p=0.0001, table S5, figure 3f) and nighttime (p<0.0001, Table S7, Figure 3g). RER was lower at night compared to daytime and the decrease in RER from day to night-time, which can be considered as a marker of metabolic flexibility, was larger after dapagliflozin as compared to placebo (-0.010 (-0.017, -0.002), p=0.016, Table S7, figure 3h). Twenty-four-hour fatty acid oxidation was higher after dapagliflozin treatment

(19.70 (12.48, 26.92) g/day, p<0.0001, Table S7, Figure 4a), resulting in a negative fat balance after dapagliflozin treatment (Figure 4b). The higher fat oxidation was observed both during daytime (20.19 (12.57, 27.81) g/day, p<0.0001, Table S7) and night-time (22.04 (13.83, 30.26) g/day, p<0.0001, Table S7). Consequently, 24h carbohydrate oxidation was lower after dapagliflozin treatment (-49.39 (-69.02, -29.77) g/day, p<0.0001, Table S7, Figure 4c) and carbohydrate balance, corrected for urinary glucose loss, remained positive during both treatment periods (Figure 4b). A lower carbohydrate oxidation after dapagliflozin treatment was observed both during daytime (-53.35 (-75.68, -31.03) g/day, p<0.0001, Table S7) and nighttime (-47.69 (-67.64, -27.74) g/day, p<0.0001, Table S7). Twenty-four-hour protein oxidation was not significantly affected by dapagliflozin treatment versus placebo (-0.91 (-7.30, 5.47) g/day, p=0.77, Table S7, Figure 4d) and did not differ between treatment periods neither during daytime (-1.99 (-8.86, 4.87) g/day, p=0.55, Table S7) nor during nighttime (-0.192 (-8.40, 8.04) g/day, p=0.96, Table S7).

While in the respiration chamber, blood was sampled at 7 times during the day. Fasting glucose levels were lower after dapagliflozin (p<0.0001, Table S5), and the area under the curve (AUC) for the plasma glucose profile during the day was significantly lower with dapagliflozin treatment (p=0.0006, Table S5, Figure 4e). Fasting NEFA levels were unaffected after dapagliflozin treatment (p=0.22, Table S5), however, AUC for the plasma NEFA profile during the day was higher after dapagliflozin (p=0.0026, Table S5, Figure 4f). Fasting (p=0.045, Table S5) and AUC of plasma levels of β-hydroxybutyrate levels during the day were higher after dapagliflozin treatment (p=0.047, Table S5, Figure 4g). AUC for plasma FGF21 was not different with dapagliflozin (p=0.16, Table S5), while AUC for plasma glucagon was higher with dapagliflozin treatment (p=0.039, Table S5).

No serious adverse events, adverse events leading to discontinuation or events of diabetic ketoacidosis were reported. One suspected diabetic ketoacidosis was diagnosed as a gastroenteritis that was not regarded as due to study treatment.

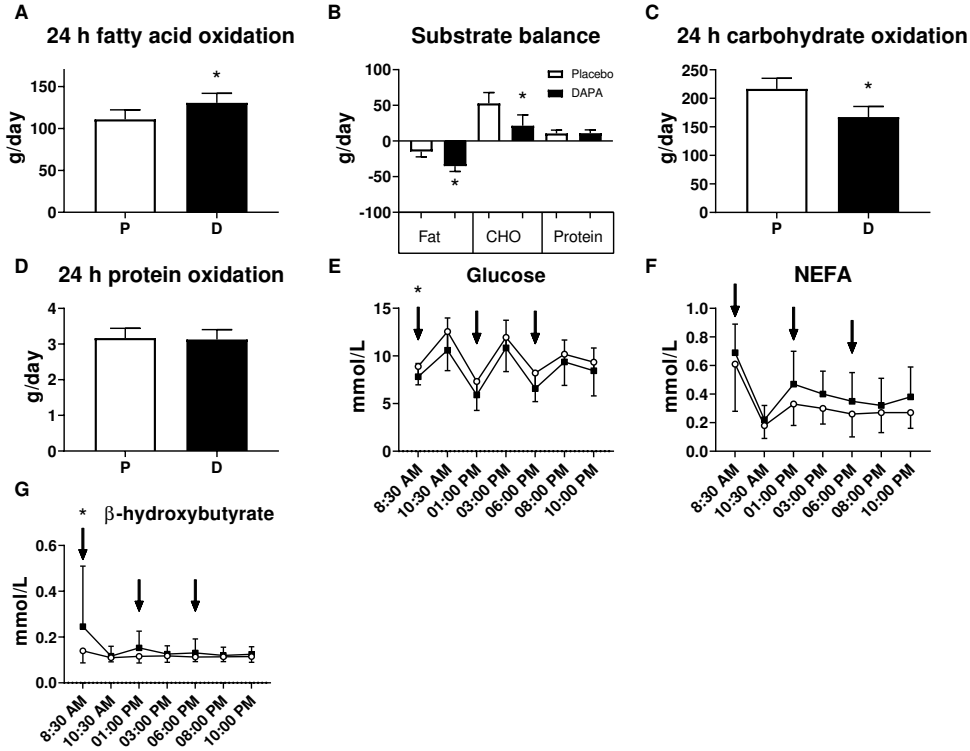

Figure 4. a) 24h fatty acid (FA) oxidation measured during the stay in the respiration chamber after Placebo (P) or dapagliflozin (D) treatment. b) substrate balance (n=24), c) 24h carbohydrate (CHO) oxidation (n=24), d) 24h protein oxidation (n=24), e) plasma glucose (n=21), f) non-esterified fatty acid (NEFA) (n=23), and g) β-hydroxybutyrate levels as measured in the respiration chamber (n=23). Blood draws at 08:30 AM, 01:00 PM and 06:00 PM were taken before meals. ○ = placebo and ■ = dapagliflozin. Results are in least squares mean (LSM) and 95% confidence interval (CI), obtained through a linear mixed model. * $P<0.05$ is considered significantly different

DISCUSSION

SGLT2 inhibitors are indicated for glucose control in type 2 diabetes and have recently been shown to have unanticipated favorable effects on CV and renal outcomes (2, 3, 24-26). The primary pharmacological action of SGLT2i cause glucose and energy loss in urine, which would mimic daily mild caloric restriction but also result in extra night-time loss of energy. Here, we demonstrate that dapagliflozin has beneficial effects on 24h energy and substrate metabolism, that mimic the effects previously observed with mild caloric restriction or time-restricted feeding (12, 27, 28).

We confirm in a controlled setting the pharmacological action of SGLT2 inhibitors, leading to a loss of urinary glucose of approximately 90 g/day. Interestingly, the rate of urinary glucose loss appeared to be twice as large during daytime compared to nighttime, which is a novel finding in this study. A plausible explanation for the differences in daytime and nighttime glucose excretion is the diurnal change in dapagliflozin concentration and therefore lower night-time concentration of dapagliflozin (29). Furthermore, higher (dietary) carbohydrate availability and higher plasma glucose levels or GFR during daytime may contribute to larger urinary glucose loss. We also observed higher glucose excretion rates during the insulin phase compared to basal phase of the glucose clamp, suggesting that also higher daytime insulin levels may be involved.

A glucose loss of 90 g/day would result in an energy deficit of ~ 1500 kJ/day and an expected decrease in body weight of ~ 1.5 to 2.0 kg during the treatment period, which is close to what we report here. However, there was no significant effect of dapagliflozin treatment on body fat, but a small but significant decrease in lean body mass, which most likely reflects a loss of extracellular water, as recently reported after 14 days of dapagliflozin treatment (30). Interestingly, we found that SGLT2i did not result in compensatory reductions in 24h energy expenditure, sleeping metabolic rate or diet-induced thermogenesis. As food intake and activity was kept constant while in the respiration chamber, overall 24h energy balance was negative after dapagliflozin treatment, inducing a state of caloric restriction. By design we provided a diet relatively high in carbohydrate, and overall 24h carbohydrate balance did not become negative by dapagliflozin treatment. This suggests that the increase in 24h fat oxidation cannot solely be attributed to a lack of substrate availability for glucose oxidation, as it is well known that the intake of carbohydrates stimulate their own oxidation (31).

However, in the postabsorptive state – specifically during the night when energy and carbohydrate balance is per definition negative – dapagliflozin may have resulted in a more pronounced negative energy balance. Indeed, a novel finding is that fat oxidation was more strongly increased at night-time, leading to a distinct fed-to-fasting transition with dapagliflozin treatment. These findings are interesting, as we have recently shown that the 24h variability in substrate oxidation – as reflected by measurement of 24h RER – is markedly blunted in volunteers with prediabetes compared to lean, healthy volunteers. This suggests that in prediabetes the diurnal oscillations in fed-fasting cycle are blunted (32), but similar measurements have not been done in patients with type 2 diabetes. However, it has been shown in type 2 diabetes that 24h fluctuations in glycogen content are blunted (31), and it is tempting to speculate that dapagliflozin helps to restore the 24h rhythmicity in glycogen metabolism since reduced glycogen levels trigger an increased fat oxidation. Moreover, a typical fed-fasting cycle, with alternating periods of energy excess and restriction, triggers a cascade of molecular pathways that are involved in the maintenance of optimal cellular functioning and thereby affect whole-body metabolic health. Indeed, inducing alternating periods of transient energy deficit also characterize interventions like exercise training, caloric restriction or time-restricted feeding, which all have been shown to improve cardiometabolic health (27, 28, 33). Thus, the beneficial health effects of dapagliflozin in type 2 diabetes may be due to the restoration of a blunted fed-to-fasting transition. Future investigations including molecular analysis of underlying mechanisms are needed to further investigate this finding.

In the current study, we did not find a beneficial effect of SGLT2 inhibitors on peripheral insulin sensitivity. Lack of improvement in peripheral insulin sensitivity was also observed by Latva-Rasku et al.(10), whereas other studies with SGLT2 inhibitors reported improvements in peripheral insulin sensitivity (8, 9, 34). Possible explanations for the discrepant findings include differences in baseline glucose control of the patients and different rates of insulin infusion. In the present study, patients' baseline HbA_{1c} level was 6.9% (51.7 mmol/mol), which is lower than reported in studies that did show an improved peripheral insulin sensitivity, in which baseline HbA_{1c} levels averaged 7.5% (58 mmol/mol) (34), 8.4-8.8% (68-73 mmol/mol) (8) and 8.5% (69 mmol/mol) (9). Worse baseline glucose control would result in larger improvement in glucose control by the intervention and therefore reduced glucotoxicity that could help to explain improved insulin sensitivity. Furthermore, the insulin infusion rate during the high-insulin phase of

the glucose clamp in our study (40 mU/m²/min) was lower compared to the infusion rate in those studies that did show improved insulin sensitivity; 80 mU/m²/min (8, 9), and 120 mU/m²/min (34). Although the insulin infusion in our study tended to suppress fat oxidation more during dapagliflozin treatment compared to placebo, the lower insulin infusion rate of 40mU/m²/min did not completely suppress fat oxidation and absolute values for fat oxidation during the clamp remained higher with dapagliflozin treatment compared to placebo; this elevated fat oxidation could impair skeletal muscle glucose uptake via substrate competition. It could be speculated that a higher insulin infusion rate would have completely inhibited fatty acid oxidation and resulted in increased insulin-mediated glucose uptake following dapagliflozin treatment. Similar changes have been observed in athletes in morning after running a marathon when insulin sensitivity was reduced and, fat oxidation and plasma NEFA levels elevated, probably due to the negative energy balance. The higher fat oxidation and lower carbohydrate oxidation in the fasting state, and the unchanged fasting R_d corrected for urinary glucose loss also led to the unexpected observation of increased NOGD in the fasted state. The cause of the increase in fasting NOGD is unclear at present unclear and needs to be repeated in subsequent studies.

In line with previous studies investigating the effect of SGLT2i on EGP (8, 9), an increased EGP in the overnight fasted state of similar magnitude as the urinary glucose excretion rate was observed. The liver is the main organ responsible for EGP and is dependent on the combined effects of glycogenolysis and gluconeogenesis. We did not determine glycogenolysis but enhanced protein oxidation upon SGLT2 inhibition has been suggested to contribute to the elevated gluconeogenesis – and thereby elevated EGP (14). However, in the current study protein oxidation based on urinary nitrogen excretion was not affected by dapagliflozin treatment and therefore a major contribution of glycogenolysis to EGP can not be excluded. Alternatively, gluconeogenesis can be fuelled by adipose tissue lipolysis and glycerol could become a major contributor of carbon for gluconeogenesis (31). Interestingly, dapagliflozin improved adipose tissue insulin sensitivity towards suppression of lipolysis reflected in larger suppression of NEFA and glycerol during insulin stimulation, which was paralleled by enhanced suppression of EGP. These novel findings indicate regulation of gluconeogenesis by dapagliflozin via changed adipose tissue insulin sensitivity and suggest that the enhanced hepatic insulin sensitivity reported here and previously (36) can be explained by improved adipose tissue insulin sensitivity. In addition, improved adipose tissue insulin sensitivity towards

suppression of lipolysis, together with enhanced fat oxidation suggests an increased fatty acid turnover, which may also explain the lower IHL content found in this and previous studies (10, 36), despite higher fasting and daytime free fatty acid levels. As IHL content is known to correlate with hepatic insulin sensitivity (37), enhanced fatty acid turnover may be another factor contributing to improved hepatic insulin sensitivity.

A limitation of the study is the short duration of treatment. Patients receiving SGLT2 inhibition for 6 months have been shown to increase food intake to compensate for the glucose losses (38). It has been suggested that the increased compensatory food intake resulting in stabilisation of weight occur approximately 2-3 months after initiation of treatment with SGLT2i (39). Therefore, the metabolic adaptations described in this study may look differently after 3 months of treatment. Furthermore, patients included in this study were well controlled and had relatively low HbA_{1C}, so effects of SGLT2 inhibition on HbA_{1c} and glucose control are limited in this study. In addition, the better glucose control of volunteers in this study may have affected the results such as effects of treatment on peripheral insulin sensitivity and limits direct comparison to studies that included less well-controlled patients.

To summarize, dapagliflozin treatment for 5 weeks resulted in caloric restriction like effects on 24h substrate flexibility, as a result of increased urinary glucose loss. Consistent with a caloric restriction-like effect, dapagliflozin also reduced hepatic lipid content and improved hepatic and adipose tissue insulin sensitivity towards suppression of lipolysis. These results may provide new insights in the mechanisms underlying the beneficial health effects of SGLT2i, although further studies are needed to provide support for this hypothesis.

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Declaration of interest

This clinical trial was funded by AstraZeneca. R.E. and J.O. are employed by AstraZeneca and AstraZeneca shareholders. All other authors declare no competing interests.

Author contribution

P.S., V.S.H., M.H., J.H., E.P., B.H., J.O. and R.E. designed and conceived the study. Y.K., M.L., B.D. E.P., J.H., M.H., V.S.H., and P.S. designed and performed the experiments. Y.K., M.L., B.D., E.P., V.S.H., P.S. analyzed the data. Y.K., P.S. and J.O. drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

Data sharing

Data underlying the findings described in this manuscript may be available upon request in accordance with AstraZeneca's data sharing policy described at https://astrazenecagroup-dt.pharmacm.com/DT/Home

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SUPPLEMENTARY DATA

Randomisation

As patients became eligible for randomization, randomization codes were assigned strictly sequentially to assign each patient to a specific treatment sequence (dapagliflozin:placebo or placebo:dapagliflozin). The randomization schedule assigned sequences using blocks of equal and fixed size, with blocks permuted and sequence randomized. An external partner (PAREXEL Ltd) provided the initial system randomization code, dummy list generation and emergency code break support.

Blood sampling scheme in respiration chamber

During the stay in the respiration chamber, blood samples were drawn at seven timepoints and used for measuring insulin, glucagon, glucose, NEFA, β-hydroxybutyrate and FGF21. The exact timing of the blood sampling is provided in table S2.

IHL determination

After an overnight fast in the respiration chamber, proton magnetic resonance spectroscopy (MRS) was used to quantify intrahepatic lipid (IHL) content. Measurements were performed on a 3.0-Tesla whole body MR system (Achieva 3Tx; Philips Healthcare), as previously described (1), however with a voxel size of 20x20x20 mm. IHL concentrations were expressed as ratios of the CH_2 peak relative to the unsuppressed water resonance (as percentage). T_2 relaxation times of 59.10ms for methylene peaks and 26.30ms for water were used.

Biochemical analysis

Lactate (Roche, Basel, Switserland), glycerol (Sigma, Saint Louis, Missouri, USA), NEFA (WAKO, Neuss, Germany) and β-hydroxybutyrate (Stanbio Laboratories) were analyzed enzymatically in serum samples using a Pentra 400 (Horiba). Insulin (Beckman Coulter Inc.) and FGF21 (R&D Systems inc.) were measured in EDTA plasma samples using immunoenzymatic assays. Glucagon (Millipore Corporation / LINCO Research) was measured in EDTA plasma samples using a radioimmunoassay. Urinary nitrogen was measured with an enzymatic colorimetric assay. Uric acid was measured in serum using enzymatic analysis using COBAS (Roche diagnostics, Indianapolis, USA). HbA_{1c} was determined by HPLC (Bio-rad, Hercules, CA, USA). High-sensitivity C-reactive protein was measured by immunonephelometry using the Siemens BNII Nephelometer (Siemens Healthcare diagnostics, Deerfield, USA).

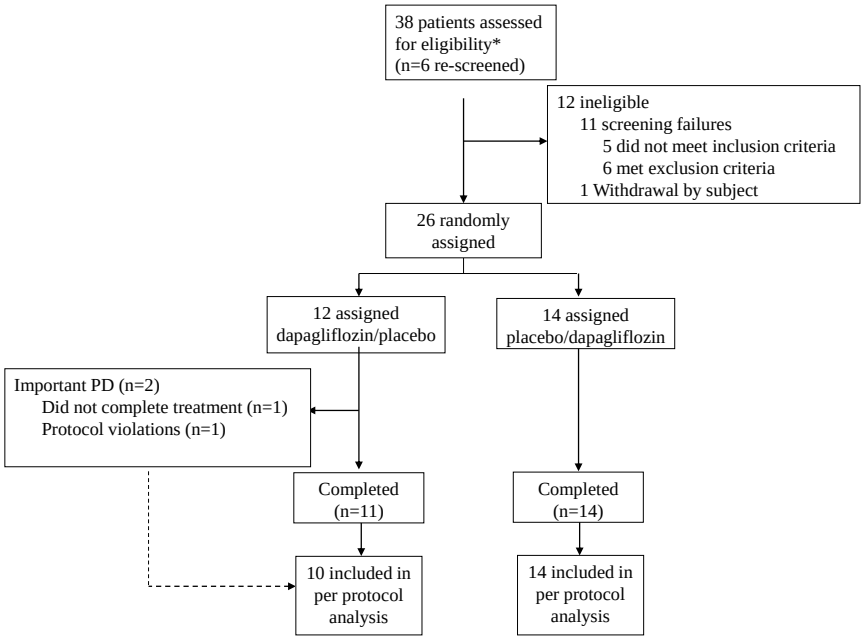

Figure S1. Study charter.

Table S1. Inclusion and exclusion criteria of the trial

Inclusion criteria	
1	Patients were able to provide signed and dated written informed consent prior to any study specific procedures.
2	Women were post-menopausal (defined as at least 1 year post cessation of menses) and aged ≥45 and ≤70 years. Males were aged ≥40 years and ≤70 years. Patients were to have suitable veins for cannulation or repeated venipuncture
3	Patients diagnosed with T2DM for at least the previous 6 months, based on American Diabetes Association 2016 standards
4	Patients were on no other anti-diabetic drug treatment, or on stable maximum 3000 mg daily dose metformin treatment and/or on stable dose of a DPPIV inhibitor treatment for at least the prior 3 months
5	HbA_{1c} levels ≥6.0% (42 mmol/mol) and ≤9.0% (75 mmol/mol).
6	Body mass index (BMI) ≤38 kg/m2
Exclusion criteria	
1	Involvement in the planning and conduct of the study (applicable to both AstraZeneca staff and staff at third party vendor or at the investigational sites).

2	Previous enrolment in the present study or participation in another clinical study with an investigational product (IP) during the previous 3 months or as judged by the Investigator
3	History of or presence of any clinically significant disease or disorder including a recent (<3 months) cardiovascular event which, in the opinion of the Investigator, may have either put the patient at risk because of participation in the study or influence the results or the patient's ability to participate in the study.
4	Clinical diagnosis of Type 1 diabetes, maturity onset diabetes of the young, secondary diabetes, or diabetes insipidus.
5	Unstable/rapidly progressing renal disease or estimated glomerular filtration rate <60 mL/min (Cockcroft-Gault formula). Males: Creatinine clearance (mL/min) = $\frac{Weight(kg)x(140-Age)}{Serumcreatine(\mu mol/L)}$ x 1.23 Females: Creatine clearance (mL/min) = $\frac{Weight(kg)(140-Age)}{Serumcreatine(\mu mol/L)}$ x 1.04
6	Clinically significant out of range values of serum levels of either alanine aminotransferase (ALT), aspartate aminotransferase (AST) or alkaline phosphatase (ALP) in the Investigator's opinion.
7	Contraindications to dapagliflozin according to the local label.
8	Use of antidiabetic drugs other than metformin or DPPIV inhibitor treatment within 3 months prior to screening.
9	Weight gain or loss >5 kg in the previous 3 months, ongoing weight loss diet (hypocaloric diet) or use of weight loss agents
10	History of drug abuse or alcohol abuse in the previous 12 months. Alcohol abuse is defined as >14 drinks per week for women and >21 drinks per week for men (1 drink = 35 cL beer, 14 cL wine, or 4 cL hard liquor), or as judged by the Investigator.
11	Any clinically significant abnormalities in clinical chemistry, hematology, or urinalysis or other condition the Investigator believes would interfere with the patient's ability to provide informed consent, comply with study instructions, or which might confound the interpretation of the study results or put the patient at undue risk.
12	Plasma donation within 1 month of screening or any blood donation/blood loss >500 mL within 3 months prior to screening or during the study.
13	Anemia defined as hemoglobin (Hb) <115 g/L (7.1 mM) in women and <120 g/L (7.5 mM) in men.
14	Use of anti-coagulant treatment such as heparin, warfarin, platelet inhibitors, thrombin, and factor X inhibitors.
15	Use of medication such as oral glucocorticoids, anti-estrogens, or other medications that are known to markedly influence insulin sensitivity.
16	Use of loop diuretics.
17	Regular smoking and other regular nicotine use.
18	Any contraindication to magnetic resonance imaging scanning. These contraindications included patients with following devices: - Central nervous system aneurysm clip - Implanted neural stimulator - Implanted cardiac pacemaker or defibrillator - Cochlear implant - Metal containing corpora aliena in the eye or brain

19	Patients who did not want to be informed about unexpected medical findings, or did not wish that their physician be informed about coincidental findings, could not participate in the study.

Calculations

Cockcroft-Gault formula was used to calculate eGFR, see table S1 for the formula.

Table S2. Timing of blood draws during the metabolic chamber measurements

Timepoint	
08:30 AM	Immediately before breakfast
10:30 AM	2 hr after breakfast
01:00 PM	Immediately before lunch
03:00 PM	2 hr after lunch
06:00 PM	Immediately before dinner
08:00 PM	2 hr after dinner
10:00 PM	4 hr after dinner

Power calculation

The power of the study was based on an anticipated effect of insulin sensitivity measured as rate of glucose disposal (R_d) corrected for glucose losses. Based on Mudaliar et. al. 2014 (2), it was assumed that R_d in subjects treated with dapagliflozin 10 mg is 17.5% greater than in participants treated with placebo (2). Earlier studies in our lab show an average R_d of 18.8 μmol/kg/min and an intra-individual standard variation of 3.53 μmol/kg/min in Rd in patients with T2DM. Inter and intra-individual variability was assumed to be equal. nQuery software calculated an estimated 22 subjects, within a two-group cross-over design, which provides approximately 80% statistical power, when using a treatment difference of 3.29 μmol/kg/min (17.5% of 18.8 μmol/kg/min) in R_d, with a two-sided alpha level of 0.05. Considering a 15% drop-out rate, a total of 26 subjects (13 subjects per sequence) were randomized. The primary outcome was defined as the difference in R_d between basal and high-rate insulin infusion.

RESULTS

Table S3. Subject characteristics

Characteristic	**Total (n=24)**
Age, years (mean ± SD)	64.2 ± 4.6
Sex, n (male/female)	19/5
Body mass index, kg/m^2 (mean ± SD)	28.1 ± 2.4
HbA$_{1c}$, mmol/mol (mean ± SD) / % (mean ± SD)	51.7 ± 6.8 / 6.9 ± 0.6
eGFR, ml/min (mean ± SD)	141 ± 13.0
Duration of diabetes, years, (median range))	8.0 (1-15)
Metformin use, (%)	71 (17/24)
Any diabetes complications, (n, yes / no)	1/23

Table S4. Results of change in blood pressure measured after 2 weeks (visit 3/6) and end-of-treatment. Parameters are expressed in least square (LS) means and 95% CI.

	Dapagliflozin		**Placebo**		**p-value**
	Lsmean	**95% CI**	**Lsmean**	**95% CI**	
Systolic blood pressure change from baseline to week 2 of treatment, mmHg	-5.113	(-9.632, -0.594)	1.653	(-2.866, 6.172	P=0.015
Diastolic blood pressure change from baseline to week 2 of treatment, mmHg	-2.681	(-5.317, -0.045)	-0.832	(-3.468, 1.804)	P=0.33
Systolic blood pressure change from baseline to week 5 of treatment, mmHg	-8.319	(-13.152, -3.487)	-4.735	(-9.568, 0.097)	P=0.28
Diastolic blood pressure change from baseline to week 5 of treatment, mmHg	-3.318	(-6.145, -0.491)	-2.667	(-5.494, 0.160)	P=0.74

Table S5. Blood parameters measured during the stay in respiration chamber. AUC= area under curve. Parameters are expressed in least square (LS) means and 95% CI.

	Dapagliflozin		**Placebo**		**p-value**
	LS mean	**(95% CI)**	**LS mean**	**95% CI**	
hsCRP, mg/L	1.389	(0.739, 2.040)	1.166	(0.516, 1.817)	P=0.50
Urate, μmol/L	267.7	(241.4, 294.0)	324.5	(298.2, 350.7)	P<0.0001
Hemoglobin, mmol/L	8.96	(8.74, 9.18)	8.78	(8.55, 9.00)	P=0.029
Erythrocyte fraction, L/L	0.420	(0.410, 0.430)	0.416	(0.406, 0.426)	P=0.43
HbA_{1c}, %	6.89	(6.55, 7.23)	6.96	(6.62, 7.30)	P=0.33
Glucose AUC, mmol/L/hr	114.157	(99.006, 129.308)	130.647	(115.496, 145.798)	P=0.0006
β-hydroxybutyrate AUC, mmol/L/hr	1.870	(1.610, 2.129)	1.580	(1.321, 1.840)	P=0.047
Free fatty acids AUC, mmol/L/hr	5.176	(4.528, 5.824)	3.917	(3.270, 4.565)	P=0.0026
Insulin ultrasensitive AUC, mIU/L/hr	368.931	(212.083, 525.779)	442.126	(285.278, 598.974)	P=0.29
Glucagon AUC, pmol/L/hr	324.973	(273.253, 376.693)	300.098	(248.379, 351.818)	P=0.039
FGF21 AUC, ng/L/hr	3310.415	(2626.919, 3993.911)	3554.716	(2871.220, 4238.212)	P=0.16
	mean	**SD**	**mean**	**SD**	
Fasting glucose levels, mmol/L	7.82	1.39	8.89	1.92	P<0.0001
Fasting NEFA levels, mmol/L	0.69	0.20	0.61	0.33	P=0.22
Fasting β-hydroxybutyrate, mmol/L	0.25	0.26	0.14	0.05	P=0.045

Table S6. Results of specific euglycemic hyperinsulinemic clamp measured parameters. Parameters are expressed in least square (LS) means and 95% CI.

	Dapagliflozin		Placebo		p-value
	LS mean	95% CI	LS mean	95% CI	
Glucose rate of disposal basal, µmol/kg/min	10.610	(9.589, 11.630)	10.494	(9.474, 11.515)	P=0.85
Glucose rate of disposal low insulin, µmol/kg/min	8.845	(8.209, 9.481)	9.392	(8.756, 10.028)	P=0.088
Glucose rate of disposal high insulin, µmol/kg/min	19.133	(16.253, 22.014)	20.086	(17.206, 22.967)	P=0.33
Δ Glucose rate of disposal $_{(high-basal)}$, µmol/kg/min	8.523	(5.566, 11.481)	9.592	(6.634, 12.549)	P=0.30
Δ Glucose rate of disposal $_{(high-basal/SSI)}$	0.018	(0.011, 0.024)	0.020	(0.014, 0.027)	P=0.27
Glucose infusion rate, ml/hr	103.63	(86.57, 120.68)	96.96	(78.82, 115.09)	P=0.21
M-value, µmol/kg/min	18.00	(14.79, 21.20)	18.77	(15.32, 22.22)	P=0.44
Endogenous glucose production basal insulin state, µmol/kg/min	12.427	(11.548, 13.306)	10.159	(9.280, 11.038)	P<0.0001
Endogenous glucose production low insulin state, µmol/kg/min	7.771	(6.914, 8.629)	7.206	(6.349, 8.063)	P=0.25
Endogenous glucose production high insulin state, µmol/kg/min	1.625	(1.079, 2.171)	1.647	(1.101, 2.193)	P=0.93
Δ Endogenous glucose production $_{(low-basal)}$, µmol/kg/min	-4.656	(-5.494, -3.817)	-2.951	(-3.790, -2.112	P=0.0036
Δ Endogenous glucose production $_{(high-basal)}$, µmol/kg/min	-10.803	(-11.726, -9.880)	-8.512	(-9.435, -7.589)	P<0.0001
Nonoxidative glucose disposal basal insulin state, µmol/kg/min	6.836	(5.464, 8.209)	4.990	(3.617, 6.362)	P=0.012
Nonoxidative glucose disposal low insulin state, µmol/kg/min	2.919	(1.666, 4.171)	1.503	(0.250, 2.755)	P=0.10
Nonoxidative glucose disposal high insulin state, µmol/kg/min	8.592	(6.334, 10.850)	8.430	(6.172, 10.688)	P=0.87
Δ Nonoxidative glucose disposal $_{(high-basal)}$, µmol/kg/min	1.726	(-0.722, 4.174)	3.488	(1.039, 5.936)	P=0.13
Glucose excretion rate, µmol/kg/min	2.465	(2.191, 2.729)	0.004	(-0.270, 0.278)	P<0.0001
Respiratory exchange ratio basal	0.756	(0.742, 0.769)	0.782	(0.768, 0.795)	P=0.0018
Respiratory exchange ratio low insulin	0.787	(0.769, 0.804)	0.816	(0.799, 0.834)	P=0.031
Respiratory exchange ratio high insulin	0.857	(0.839, 0.874)	0.874	(0.857, 0.891)	P=0.049
Δ Respiratory exchange ratio $_{(high-basal)}$	0.101	(0.080, 0.122)	0.089	(0.068, 0.110)	P=0.18
Carbohydrate oxidation basal, µmol/kg/min	3.774	(2.843, 4.704)	5.504	(4.574, 6.435)	P=0.0016
Carbohydrate oxidation low insulin, µmol/kg/min	5.859	(4.616, 7.101)	7.888	(6.645, 9.131)	P=0.030
Carbohydrate oxidation high insulin, µmol/kg/min	10.687	(9.424, 11.950)	11.797	(10.534, 13.060)	P=0.071
Δ Carbohydrate oxidation $_{high-basal}$, µmol/kg/min	6.910	(5.472, 8.349)	6.139	(4.700, 7.577)	P=0.17
Fat oxidation basal, µmol/kg/min	4.064	(3.807, 4.322)	3.535	(3.277, 3.792)	P=0.0022
Fat oxidation low insulin, µmol/kg/min	3.454	(3.169, 3.739)	2.957	(2.672, 3.242)	P=0.015
Fat oxidation high insulin, µmol/kg/min	2.309	(2.018, 2.599)	2.011	(1.720, 2.301)	P=0.055
Δ fat oxidation $_{high-basal}$, µmol/kg/min	-1.783	(-2.148, -1.418)	-1.501	(-1.866, -1.137)	P=0.13

Insulin levels basal state, pmol/L	43.12	(34.73, 51.51)	61.29	(52.90, 69.68)	P<0.0001
Insulin levels low-insulin state, pmol/L	124.71	(107.91, 141.51)	145.43	(128.64, 162.23)	P=0.019
Insulin levels high-insulin state, pmol/L	507.93	(469.31, 546.54)	517.65	(479.04, 556.27)	P=0.46
NEFA levels basal, μmol/L	799.82	(667.75, 931.88)	590.94	(455.29, 726.59)	P=0.011
NEFA levels low-insulin state, μmol/L	421.16	(352.11, 490.20)	368.96	(299.91, 438.00)	P=0.076
NEFA levels high-insulin state, μmol/L	223.85	(148.82, 298.87)	277.06	(202.03, 352.08)	P=0.031
Suppression of NEFA low-insulin state, % suppression from basal	-39.67	(-58.92, -20.43)	-27.91	(-47.41, -8.41)	P=0.12
Suppression of NEFA high-insulin state, % suppression from basal	-70.84	(-85.80, -55.88)	-48.91	(-64.28, -33.55)	P=0.016
Glycerol levels basal, mmol/L	0.067	(0.054, 0.081)	0.046	(0.032, 0.059)	P=0.013
Glycerol levels low-insulin state, mmol/L	0.027	(0.019, 0.035)	0.036	(0.028, 0.044)	P=0.10
Glycerol levels high-insulin state, mmol/L	0.032	(0.025, 0.040)	0.039	(0.031, 0.047)	P=0.16
Δ **glycerol low-basal**, mmol/L	-0.040	(-0.056, -0.025)	-0.010	(-0.026, 0.006)	P=0.010
Δ **glycerol high-basal**, mmol/L	-0.036	(-0.050, -0.021)	-0.007	(-0.021, 0.008)	P=0.009
Lactate levels basal state, mmol/L	2.00	(1.80, 2.20)	2.13	(1.93, 2.33)	P=0.26
Lactate levels low-insulin state, mmol/L	1.70	(1.54, 1.87)	1.90	(1.73, 2.06)	P=0.032
Lactate levels high-insulin state, mmol/L	1.73	(1.61, 1.86)	1.84	(1.71, 1.96)	P=0.092

Table S7. Results of parameters measured during the stay in the respiration chamber. Parameters are expressed in least square (LS) means and 95% CI.

	dapagliflozin		**Placebo**		**p-value**
	LS mean	**95% CI**	**LS mean**	**95% CI**	
24h energy expenditure, MJ/day	9.519	(9.017, 10.020)	9.628	(9.126, 10.130)	P=0.11
Sleeping metabolic rate, MJ/day	6.571	(6.251, 6.891)	6.621	(6.302, 6.941)	P=0.36
Diet induced thermogenesis, MJ/day	1.279	(1.102, 1.456)	1.317	(1.140, 1.493)	P=0.64
24h respiratory exchange ratio	0.812	(0.803, 0.821)	0.835	(0.826, 0.844)	P=0.0001
Respiratory exchange ratio day time	0.817	(0.807, 0.827)	0.841	(0.831, 0.851)	P=0.0001
Respiratory exchange ratio night time	0.797	(0.786, 0.807)	0.830	(0.819, 0.840)	P<0.0001
Δrespiratory exchange ratio $_{(day-night)}$	0.021	(0.013, 0.028)	0.011	(0.003, 0.019)	P=0.016
Protein oxidation 24h, g/day	75.096	(68.448, 81.720)	76.008	(69.360, 82.632)	p=0.77
Protein oxidation day time, g/day	82.272	(74.880, 89.664)	84.264	(76.872, 91.656)	P=0.55
Protein oxidation night time, g/day	61.464	(53.592, 69.312)	61.632	(53.784, 69.504)	P=0.96
Carbohydrate oxidation 24h, g/day	167.12	(1478.58, 185.65)	216.51	(197.98, 235.04)	P<0.0001
Carbohydrate oxidation daytime, g/day	204.09	(180.61, 228.56)	257.44	(233.97, 280.91)	P<0.0001
Carbohydrate oxidation nighttime, g/day	105.39	(90.01, 120.78)	153.08	(137.70, 168.47)	P<0.0001
Fat oxidation 24h, g/day	130.50	(119.08, 141.93)	110.80	(99.38, 122.23)	P<0.0001
Fat oxidation day time, g/day	142.09	(128.51, 155.68)	121.91	(108.32, 135.49)	P<0.0001
Fat oxidation night time, g/day	107.34	(97.77, 116.90)	85.29	(75.73, 94.86)	P<0.0001
Glucose excretion 24h, mmol/day	510.7	(434.8, 586.7)	40.7	(-35.2, 116.7)	P<0.0001
Glucose excretion daytime, mmol/hr	23.98	(20.41, 27.56)	2.15	(-1.42, 5.72)	P<0.0001
Glucose excretion nighttime, mmol/hr	10.99	(8.80, 13.19)	0.30	(-1.89, 2.50)	P<0.0001
Energy balance, MJ/day	0.47	(0.23, 0.71)	0.38	(0.13, 0.62)	P=0.34
Energy balance corrected for urinary glucose loss, MJ/day	-1.10	(-1.41, -0.78)	0.26	(-0.05, 0.57)	P<0.0001
Protein intake, g/day	86.02	(80.67, 91.38)	87.03	(81.59, 92.46)	N.A.
Carbohydrate intake, g/day	277.44	(258.10, 296.78)	275.74	(257.91, 293.57)	N.A.
Fat intake, g/day	95.26	(88.26, 102.26)	96.07	89.16, 102.99)	N.A.
Protein balance, g/day	10.92	(6.27, 15.57)	10.61	(5.96, 15.26)	P=0.91
Carbohydrate balance corrected for urinary glucose loss, g/day	21.35	(6.04, 36.66)	52.68	(37.37, 67.99)	P=0.0003
Fat balance, g/day	-35.24	(-42.77, -27.72)	-14.73	(-22.26, -7.21)	P<0.0001
Activity levels, counts/min	199.48	(179.03, 219.94)	193.15	(172.69, 213.61)	P=0.42

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Chapter 7

Treatment with SGLT2 inhibitor dapagliflozin alters skeletal muscle acylcarnitine species and increases IMCL in patients with type 2 diabetes

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ABSTRACT

Background: SGLT2 inhibitors increase urinary glucose excretion and have beneficial effects on cardiovascular and renal outcomes; the underlying mechanism may involve caloric restriction-like metabolic effects due to urinary glucose loss. We investigated the effects of dapagliflozin on skeletal muscle mitochondrial function and metabolism-derived substrate intermediates.

Methods: Twenty-six type 2 diabetes mellitus patients were randomized to a 5-week double-blind, cross-over study with 6-8-week wash-out. Skeletal muscle acetylcarnitine levels, intramyocellular lipid (IMCL) content and phosphocreatine (PCr) recovery rate were measured by magnetic resonance spectroscopy (MRS). *Ex vivo* mitochondrial respiration was measured in skeletal muscle fibers using high resolution respirometry. Skeletal muscle levels of acylcarnitines, amino acids and TCA cycle intermediates were measured. Results are presented as the least squares mean (95% CI) difference between treatment arms.

Results: Evaluable patients (n=24) had unaltered *in vivo* mitochondrial function, measured as PCr recovery with ^{31}P-MRS, and *ex vivo* mitochondrial respiration in skeletal muscle fibers after dapagliflozin treatment. Skeletal muscle acetylcarnitine levels prior and after 30 minutes of exercise remained unaltered after dapagliflozin treatment, as well as the maximum capacity to form acetylcarnitine upon exercise. Citrate synthase and carnitine acetyltransferase activities were not affected by dapagliflozin. Dapagliflozin treatment increased IMCL content, as measured by ^{1}H-MRS (0.060 (0.011, 0.110) %, p=0.019). Several acylcarnitine species (mostly long-chain) and C4-OH carnitine levels (0.4704 (0.1246, 0.8162) pmoles*mg tissue^{-1}, p<0.001) in skeletal muscle were higher after dapagliflozin treatment, while C2 carnitine levels were lower (-40.0774 (-64.4766, -15.6782) pmoles*mg tissue^{-1}, p<0.001). Several amino acids (alanine, proline, valine and glutamic acid) in skeletal muscle were lower after dapagliflozin treatment. Also succinate, alpha-ketoglutarate and lactate were significantly lower after dapagliflozin treatment.

Conclusion: Dapagliflozin treatment for 5 weeks changed acylcarnitine species reflecting higher skeletal muscle lipid oxidation rates, while IMCL content increased suggesting a metabolic remodeling of skeletal muscle more closely reflecting a healthy, metabolically flexible muscle phenotype.

INTRODUCTION

Sodium-glucose cotransporter 2 inhibitors (SGLT2i) inhibit renal glucose reabsorption and increase urinary glucose excretion. Improved glycemic control, reduced cardiovascular (CV) risk, including reduced hospitalization for heart failure and reduced risk of kidney disease progression have been reported with SGLT2i treatment (1, 2). The primary action of SGLT2i on glucose and sodium reabsorption has several metabolic consequences that may help to explain the effects on CV and renal outcome (3). Such metabolic effects may reflect the adaptive response to the loss of about 50-100g glucose per day in the urine, resulting in a form of mild caloric restriction and caloric loss overnight. In line, recently we showed that 5 weeks of SGLT2i treatment in type 2 diabetes patients led to whole body, 24h metabolic adaptations that mimic the effects of calorie restriction, such as improved 24h fat oxidation, improved hepatic and adipose tissue insulin sensitivity and improved 24h energy metabolism (4). The present findings were in line and extended previous reports of increased fat oxidation (5), decreased intrahepatic lipid (IHL) content (6), decreased total fat mass (3, 7), and decreased visceral adipose tissue (7) after SGLT2 inhibitor treatment in type 2 diabetes patients.

Calorie restriction like effects in humans are accompanied by adaptations in skeletal muscle metabolism, which can underly metabolic health effects of such interventions. Thus, calorie restriction improves skeletal muscle fat oxidative and mitochondrial capacity (8). Such adaptations are important, as we have previously shown that high whole-body and mitochondrial lipid oxidation capacity attenuates lipotoxicity (9). Furthermore, lipid-induced insulin resistance in skeletal muscle is associated with reduced mitochondrial function (10-12). Indeed, under diabetogenic conditions, lipid supply to skeletal muscle may exceed mitochondrial oxidative capacity, resulting in the accumulation of intramyocellular lipids causing lipotoxicity and thereby insulin resistance. It has been suggested that carnitine acetyltransferase (CrAT) can function as a defensive mechanism against such mitochondrial substrate oversupply. Thus, excessive mitochondrial acetyl-CoA can be converted by CrAT to acetylcarnitine (13) and thereby reduce the allosteric inhibition on pyruvate dehydrogenase complex and subsequently increase mitochondrial glucose oxidation (14). Alleviating substrate competition on the level of mitochondria (15, 16), could improve metabolic flexibility in patients with T2DM. Indeed, the capacity to form acetylcarnitine in skeletal muscle has been suggested to be a determinant of insulin sensitivity (17, 18).

Whether SGLT2 inhibition exerts effects on skeletal muscle metabolism, such as improved fat oxidative capacity and improved intramyocellular lipid metabolism, that could underly the metabolic health effects of this novel class of anti-diabetes drugs, is so far largely unknown. Daniele et al. reported that SGLT2 inhibitor treatment reduces global ATP synthesis rate in skeletal muscle, suggesting a shift of acetyl-CoA metabolism towards ketone production, rather than oxidation in the TCA cycle (5). Here we investigated the hypothesis that SGLT2 inhibition induces caloric restriction-like effects, and subsequently alleviates lipid-induced mitochondrial dysfunction and substrate competition on the mitochondrial level. To this end, we investigated the effect of 5 weeks of SGLT2i treatment on skeletal muscle substrate handling, and aimed to explore the effect of dapagliflozin treatment on mitochondrial function, mitochondrial substrate competition and capacity to form acetylcarnitine.

METHODS

Study design and participants

A double-blind, randomized, placebo-controlled, cross-over Phase IV trial study, was conducted at the Metabolic Research Unit Maastricht (MRUM) of Maastricht University as previously reported (4). The study took place between 5 March 2018 and 4 November 2019. The study protocol was approved by the Ethics Committee of Maastricht University Medical Center and was conducted conform to the declaration of Helsinki (19). Patients were randomized to a double-blind, placebo-controlled intervention study with treatment for 2 treatment periods, each of 5 weeks or a maximum duration of 40 days, separated by a wash-out period of 6 to 8 weeks. End-points were assessed at the end of each 5-week period. In brief, the target population consisted of patients with T2DM diagnosed for at least 6 months who had been stable on a dose of metformin and/or a DPPIV inhibitor for the previous 3 months or more or were drug naïve. Patients were to have HbA_{1c} levels between 6% and 9% (42 and 75 mmol/mol). A table with all inclusion and exclusion criteria was previously published (4). Written informed consent was obtained from all participants before inclusion.

Procedures

In short, the study comprised seven visits, including the screening (Visit 1). Visit 2 (or 5), the first visit of period 1 or 2, included randomisation of the patients and measurement of safety markers. After two weeks of treatment, a safety visit (Visit 3 or 6) followed, and an end-of-treatment visit (Visit 4 or 7) after 5 weeks of treatment. All outcome measures were performed at Visit 4/7 and were spread over a period of 6-8 days. Visit 4 was followed by a 6-8 weeks wash-out period. The primary outcome parameter was comparison of dapagliflozin versus placebo after 5 weeks treatment on skeletal muscle insulin sensitivity measured as the change in glucose disposal rate from basal to the high-insulin state and corrected for urinary glucose loss using a 2-step EHC procedure. Exploratory endpoints were whole-body 24h energy expenditure and substrate oxidation, whole-body and tissue-specific insulin sensitivity and body composition including liver fat accumulation which have been published previously (4).

Maximal VO2 cycling test

For baseline characterization (Visit 2), participants performed a graded cycling test to determine maximal oxygen uptake (VO_{2max}) and maximal power output (W_{max}) as

previously described (20).

Maximal knee-extension test

A maximal knee extension test was performed at Visit 2 to determine exercise intensity of 60% of maximal weight to be used during the phosphocreatine (PCr) recovery ^{31}P magnetic resonance spectroscopy (^{31}P-MRS) measurements scheduled for Visits 4 and 7. This test was performed on a MR- compatible knee-extension exercise device, with incremental weight put on the left leg (500 g every 30 seconds) until exhaustion (21).

Magnetic resonance spectroscopy measurements

On the first day of the end-of-treatment visit, at 3:00 P.M., *in vivo* IMCL content in tibialis anterior muscle was assessed by ^{1}H-MRS on a 3.0 T whole-body magnetic resonance system (Achieva 3Tx; Philips Healthcare) as described previously (22). The ^{1}H-MRS spectra obtained were analysed and fitted in the time domain by using the non-linear least-squares Advanced Method for Accurate, Robust, and Efficient Spectral (AMARES) algorithm (23, 24), in the java-based magnetic resonance user interface (jMRUI) software package (25) as described earlier (22). IMCL is given as percentage of the CH_2 peak compared with the water resonance and corrected for T_1 and T_2 relaxation time. IMCL could not be measured in one participant due to technical failure.

Subsequently, *in vivo* mitochondrial oxidative capacity was determined by ^{31}P-MRS at 4:00 P.M. in the afternoon, as previously described (21). A knee-extension protocol was performed for 5 minutes, on a custom-built magnetic resonance-compatible ergometer with a pulley system in a 3.0 T whole-body MRI scanner (Achieva 3Tx; Philips Healthcare). A coil (5 cm diameter) was positioned on the vastus lateralis muscle and a time series of ^{31}P-MRS spectra (free induction decays) were acquired with a repetition time of 4 seconds. Post-exercise PCr kinetics was computed as previously described (21). On a separate day, at the last day of the end-of-treatment visit to prevent effects of exercise testing on the other outcome parameters, acetylcarnitine concentrations were acquired by ^{1}H-MRS in skeletal muscle before and after exercise. Participants were fasted from 12:00 A.M. and were asked to refrain from strenuous physical activity 72h before the measurement. Resting skeletal muscle acetylcarnitine concentrations were measured at 5 P.M. using a T_1-editing method, as described previously (26), after which volunteers performed a 30 min cycling exercise at 70% maximal output on an ergometer. Immediately following exercise, acetylcarnitine concentrations were measured again.

Acetylcarnitine values were converted to absolute concentrations as described previously (17). The creatine peak was used as a reference. During the 30 minutes cycling, blood draws were performed to measure lactate levels at the start, after 15 minutes and directly after cycling (30 minutes).

Muscle biopsy

On day 3 of the end-of-treatment visit, a percutaneous muscle biopsy was obtained from the vastus lateralis muscle in the fasted state before the start of a hyperinsulinemic euglycemic clamp and as described previously (27), under local anaesthesia (1% lidocaine), as described by Bergström et al. (28). A small portion of tissue was immediately placed in preservation medium (BIOPS; Oroboros Instruments, Innsbruck, Austria). Muscle fibers were separated with small needles and the muscle membrane was permeabilized with a Saponin stock solution (5 mg/mL BIOPS), as previously described (27). Saponin was removed and ~3-4 mg wet weight fiber was transferred into the oxygraph. The remainder of the biopsy was immediately snap frozen and stored at −80°C, for assessment of carnitine acetyltransferase (CrAT) and citrate synthase (CS) activity, and levels of acylcarnitines, amino acids and organic acids. Additional skeletal muscle tissue was placed in isopentane, and then frozen in liquid nitrogen and stored at −80°C.

High-resolution respirometry

Muscle fibers were permeabilized as previously described (27). High resolution respirometry was used to measure *ex vivo* mitochondrial respiration, under hyperoxic conditions at 37°C in a two-chamber oxygraphy (Oroboros, Innsbruck, Austria) and expressed as pmol/mg muscle fiber wet weight/s. Oxidative phosphorylation was measured by adding 4.0 mmol/L malate, 10.0 mmol/L glutamate, 2.0 mmol/L ADP and 10.0 mmol/L succinate, with or without the presence of 40 µmol/L octanoylcarnitine. Leak respiration or maximal respiratory capacity was determined by adding respectively 2.0 µg/mL oligomycin or 0.5 µmol/L titrations of uncoupler fluoro-carbonyl cyanide phenylhydrazone. Cytochrome C (10.0 mmol/L) was added to check the integrity of mitochondrial outer membrane, and revealed good quality of all permeabilized mitochondrial analysis.

Biochemical analysis

Acylcarnitines, amino acids and organic acids were analyzed in skeletal muscle tissue

obtained from the muscle biopsy taken visit 4 and 7 by flow injection tandem mass spectrometry, using sample preparation methods described previously (29, 30). Skeletal muscle organic acids were measured using gas chromatography-mass spectrometry as previously described (31). Data were acquired using a Waters AcquityTM UPLC system with a TQ (triple quadrupole) detector. The data system was controlled by MassLynx 4.1 operating system (Waters, Milford, MA). Plasma levels of lactate (Roche, Basel, Switzerland) was analyzed enzymatically in EDTA samples using a Pentra 400 (Horiba).

Statistics

The evaluable analysis set, consisting of patients with at least one dose of the investigational product (per protocol) and no important protocol deviations, was used for the statistical analyses, using SAS® software version 9.04 and performed by IQVIA Biostatistics, Reading, United Kingdom. The expected difference between treatment groups was estimated using a linear mixed effects model. This model had treatment group, treatment sequence and period as fixed effects, as well as random intercept for each subject. This model assume independent conditional residuals with equal variations in each period and treatment group. Residual plots and tests for normal distribution of model residuals were used to check model assumptions. If strong deviations from normality was detected, a non-parametric test of treatment difference against zero was performed (Wilcoxon paired signed-rank test) using all the data and ignoring the sequence. The least-squares (LS) means for treatment effect in the respective treatment groups and the corresponding 95% CIs are presented. The difference in LS means between the two treatments was generated, with corresponding 95% CI and p-value tabulated. Pearson correlations are performed using a linear regression model. If deviations from normality was detected, a spearman correlation was performed. A two-sided 0.05 level is considered as statistically significant. We used the Benjamini-Hochberg procedure for multiple testing, and found that adjusted p-values were in agreement with unadjusted p-values.

The trial is registered with ClinicalTrials.gov, number NCT03338855.

RESULTS

Unaltered mitochondrial function and acetylcarnitine levels after dapagliflozin treatment

We have previously reported that dapagliflozin treatment for 5 weeks increased 24h whole-body fat oxidation in these type 2 diabetes patients (4). To investigate if these effects were associated with altered mitochondrial function, we determined *in vivo* mitochondrial function, measured as PCr recovery with ^{31}P-MRS. However, *in vivo* mitochondrial function remained unchanged upon dapagliflozin treatment (Figure 1a). Consistently, also *ex vivo* mitochondrial respiration of skeletal muscle measured after an overnight fast, remained unchanged with dapagliflozin treatment (Figure 1b). Citrate synthase activity, which reflect mitochondrial content, was not significantly affected by dapagliflozin treatment (-0.37 (-1.22, 0.48) μmol/min/gr, p=0.37). Patients were characterized with a mean (SD) VO_{2max} of 29.5 (5.6) ml O_2/kg/min. VO_{2max} correlated positively with maximum mitochondrial fat oxidative phosphorylation capacity (state U) (r=0.706, p=0.001). We previously reported that 5 weeks of dapagliflozin treatment resulted in improved day-to-night switch from glucose to fat oxidation (4). To investigate if the improved day-to-night switch from glucose to fat oxidation was related to improved acetylcarnitine metabolism, acetylcarnitine levels were determined using ^{1}H-MRS before and after 30 minutes of exercise. Acetylcarnitine levels both prior to and immediately after exercise remained unchanged with dapagliflozin treatment (Figure 1c), and also maximum capacity to form acetylcarnitine was unaffected by dapagliflozin treatment (Figure 1d and e). During the 30 minutes of exercise the increase in plasma lactate levels was lower after dapagliflozin treatment (-0.604 (-1.204, -0.004) mmol/L, p=0.048, Figure 1f).

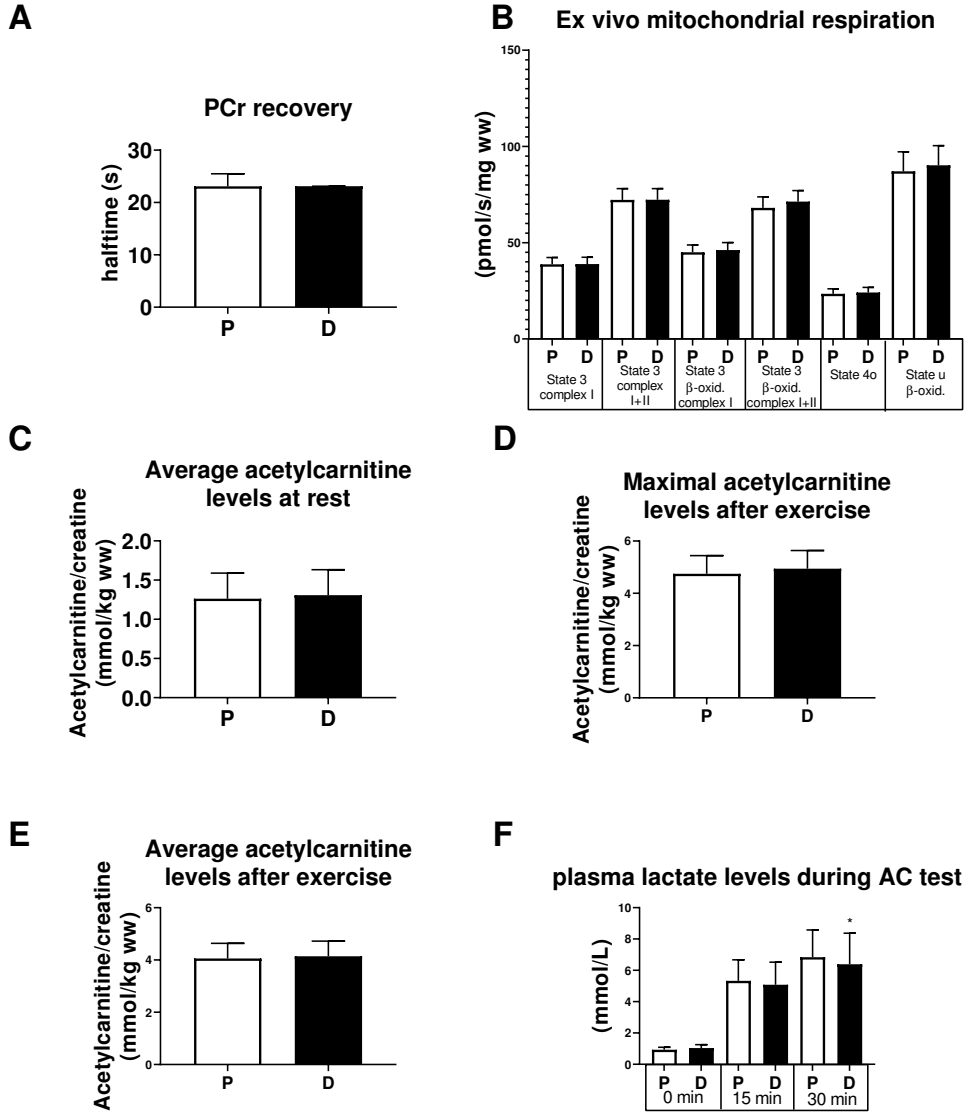

Figure 1. a) Phosphocreatine (PCr) recovery (n=22), b) *ex vivo* mitochondrial respiration (n=22), c) average acetylcarnitine levels at rest (n=23, d) maximal acetylcarnitine levels after exercise (n=21), e) average acetylcarnitine levels after exercise (n=21), and f) plasma lactate levels during exercise (n=20) after placebo (P) and dapagliflozin (D) treatment. Results are in least squares mean (LSM) and 95% confidence interval (CI), obtained through a linear mixed model. * P<0.05 is considered significantly different.

Increased IMCL content and acylcarnitine levels in muscle after dapagliflozin treatment

We next investigated if dapagliflozin treatment affected skeletal muscle lipid metabolism. We previously reported that dapagliflozin decreased trunk fat mass and hepatic lipid content (4) and hypothesized that IMCL content may be reduced as well. IMCL content correlated negatively with state U in both arms (Figure 2a and b), suggesting that a low mitochondrial capacity was associated with high IMCL content. Interestingly, IMCL content as measured by ^{1}H-MRS was significantly increased after dapagliflozin treatment (0.060 (0.011, 0.110) %, p=0.019, Figure 2c). Of 20 patients, 13 had an increase in IMCL and 2 remained similar in IMCL content, after dapagliflozin treatment. Although dapagliflozin did not significantly improve peripheral insulin sensitivity on a group level (4), the change in insulin sensitivity between both treatments showed a trend towards a positive correlation with the change in IMCL between both treatments (Figure 2d) suggesting that an increase in IMCL was associated with an improvement in insulin sensitivity.

To further investigate if the increase in IMCL upon dapagliflozin reflects a state of lipotoxicity or parallels the effects observed with exercise training and/or calorie restriction, we next investigated acylcarnitine species in muscle as a marker of muscle-specific fat oxidative capacity. Acetylcarnitine (C2) levels -measured in muscle biopsies taken after an overnight fast– were decreased upon dapagliflozin treatment (-40.0774 (-64.4766, -15.6782) pmoles*mg tissue^{-1}, p<0.001, Table 1). The decrease in acetylcarnitine levels upon dapagliflozin correlated with the increase in 24h fat oxidation (figure 2e). Carnitine acetyltransferase (CrAT) activity showed a trend towards lower activity after dapagliflozin treatment (-0.381 (-0.796, 0.033), p=0.069, Table 3). However, all other acylcarnitines metabolites were generally higher after dapagliflozin. Thus, levels of C4-OH (0.4704 (0.1246, 0.8162) pmoles*mg tissue^{-1}, p<0.001, Table 1) were significantly higher after dapagliflozin compared to placebo. Similarly, the long-chain acylcarnitines, C16:1 (0.0430 (-0.047, 0.907) pmoles*mg tissue^{-1}, p=0.042, Table 1), C18 (0.3839 (0.04121, 0.7267) pmoles*mg tissue^{-1}, p=0.013), C18:1 (1.4837 (0.08009, 2.8873) pmoles*mg tissue^{-1}, p=0.044, C18:2 (0.3939 (0.01450, 0.7733) pmoles*mg tissue^{-1}, p=0.047) and C20:1 (0.01984 (0.000427, 0.03925) pmoles*mg tissue^{-1}, p=0.027) were also higher after dapagliflozin treatment (Table 1). A complete overview of all acylcarnitines in skeletal muscle can be found in Table 1.

Table 1. Skeletal muscle levels of carnitine species, measured during the end-of-treatment visit. All parameters are in pmoles*mg tissue^{-1} and expressed in least square means and 95% CI. The p-value is obtained from a Wilcoxon signed rank test.

	Dapagliflozin		**Placebo**		**p-value**
	LSmean	**95% CI**	**LSmean**	**95% CI**	
C0	2131.03	(1867.67, 2394.38)	2263.00	(1999.65, 2526.35)	P=0.29
C2	114.56	(91.16, 137.95)	154.63	(131.24, 178.03)	P<0.001
C3	2.25	(1.86, 2.63)	2.53	(2.15, 2.92)	P=0.20
C4/Ci4	1.02	(0.84, 1.20)	0.86	(0.69, 1.04)	P=0.051
C5:1	0.41	(0.34, 0.47)	0.37	(0.31, 0.44)	P=0.54
C5	0.90	(0.68, 1.13)	0.73	(0.51, 0.95)	P=0.082
C4-OH	1.30	(0.95, 1.65)	0.83	(0.48, 1.18)	P<0.001
C6	1.18	(0.87, 1.49)	1.3	(0.71, 1.34)	P=0.21
C5-OH/C3-DC	1.70	(1.48, 1.92)	1.69	(1.48, 1.91)	P=1.00
C4-DC/Ci4-DC	1.71	(1.49, 1.92)	1.69	(1.47, 1.90)	P=0.74
C8:1	0.22	(0.18, 0.26)	0.19	(0.14, 0.23)	P=0.11
C8	0.34	(0.24, 0.45)	0.31	(0.20, 0.41)	P=0.16
C5-DC	1.21	(0.85, 1.56)	1.34	(0.98, 1.80)	P=0.52
C8:1-OH/C6:1-DC	0.054	(0.040, 0.069)	0.040	(0.026, 0.055)	P=0.22
C6-DC/C8-OH	0.18	(0.15, 0.22)	0.17	(0.14, 0.21)	P=0.64
C10:1	0.068	(0.050, 0.087)	0.052	(0.034, 0.071)	P=0.13
C10	0.27	(0.20, 0.34)	0.23	(0.16, 0.30)	P=0.065
C10-OH/C8-DC	0.067	(0.047, 0.087)	0.053	(0.033, 0.072)	P=0.33
C12:1	0.13	(0.094, 0.16)	0.092	(0.057, 0.13)	P=0.088
C12	0.39	(0.28, 0.50)	0.31	(0.20, 0.42)	P=0.27
C12-OH/C10-DC	0.045	(0.032, 0.058)	0.033	(0.020, 0.046)	P=0.24
C14:2	0.19	(0.13, 0.26)	0.14	(0.080, 0.21)	P=0.065
C14:1	0.71	(0.48, 0.94)	0.48	(0.25, 0.71)	P=0.073
C14	0.84	(0.57, 1.11)	0.57	(0.30, 0.84)	P=0.088
C14:3-OH/C12:3-DC	0.009	(0.004, 0.015)	0.008	(0.002, 0.013)	P=0.53
C14:2-OH/C12:2-DC	0.029	(0.021, 0.037)	0.020	(0.012, 0.028)	P=0.14
C14:1-OH/C12:1-DC	0.092	(0.071, 0.11)	0.066	(0.044, 0.087)	P=0.079
C14-OH/C12-DC	0.053	(0.042, 0.064)	0.058	(0.047, 0.069)	P=0.56
C16:3	0.057	(0.041, 0.073)	0.041	(0.024, 0.057)	P=0.088
C16:2	0.22	(0.15, 0.29)	0.14	(0.07, 0.21)	P=0.11
C16:1	1.10	(0.76, 1.44)	0.67	(0.33, 1.01)	P=0.043
C16	2.70	(1.97, 3.43)	1.91	(1.17, 2.63)	P=0.070
C16:2-OH/C14:2-DC	0.050	(0.036, 0.065)	0.044	(0.029, 0.058)	P=0.78
C16:1-OH/C14:1-DC	0.099	(0.072, 0.13)	0.081	(0.055, 0.11)	P=0.15
C16-OH/C14-DC	0.072	(0.056, 0.089)	0.072	(0.055, 0.088)	P=0.96
C18:3	0.14	(0.11, 0.18)	0.12	(0.087, 0.15)	P=0.22
C18:2	1.52	(1.16, 1.88)	1.13	(0.77, 1.49)	P=0.047
C18:1	4.40	(3.32, 5.48)	2.92	(1.83, 4.00)	P=0.045
C18	1.31	(1.02, 1.60)	0.92	(0.63, 1.22)	P=0.013
C18:2-OH/C16:2-DC	0.050	(0.038, 0.063)	0.040	(0.027, 0.053)	P=0.26
C18:1-OH/C16:1-DC	0.074	(0.060, 0.088)	0.061	(0.047, 0.075)	P=0.28
C20:4	0.16	(0.11, 0.21)	0.17	(0.11, 0.22)	P=0.99
C20:3	0.054	(0.039, 0.068)	0.516	(0.037, 0.066)	P=0.56
C20:2	0.045	(0.034, 0.057)	0.042	(0.031, 0.054)	P=0.92

C20:1	0.078	(0.059, 0.098)	0.059	(0.039, 0.078)	P=0.027
C20	0.040	(0.030, 0.050)	0.034	(0.024, 0.045)	P=0.41
C20:3-OH/C18:3-DC	0.058	(0.048, 0.067)	0.047	(0.038, 0.056)	P=0.12
C20:2-OH/C18:2-DC	0.049	(0.037, 0.061)	0.039	(0.027, 0.050)	P=0.25
C20-OH/C18-DC/C22:6	0.032	(0.024, 0.039)	0.034	(0.027, 0.042)	P=0.47
C22:5	0.025	(0.019, 0.031)	0.030	(0.024, 0.036)	P=0.19
C22:1	0.032	(0.020, 0.044)	0.042	(0.031, 0.054)	P=0.27
C22	0.026	(0.019, 0.032)	0.030	(0.023, 0.036)	P=0.53

Lower amino acids and TCA cycle intermediates in muscle after dapagliflozin treatment

The effects of dapagliflozin on IMCL and acylcarnitine profiles are consistent with beneficial effects of dapagliflozin on skeletal muscle fat oxidative capacity, which are also observed after interventions such as exercise training and calorie restriction. To further investigate if dapagliflozin affected skeletal muscle oxidative metabolism, we also measured levels of amino acids and organic acids in muscle tissue from the muscle biopsy. We found that alanine (-150.16 (-245.32, -55.0091) pmoles*mg tissue^{-1}, p=0.013), proline (-46.0350 (-78.7122, -13.3578) pmoles*mg tissue^{-1}, p=0.0094), valine (-14.6018 (-26.1708, -3.0327) pmoles*mg tissue^{-1}, p=0.0094), and glutamic acid (-166.71 (-299.82, -33.6025) pmoles*mg tissue^{-1}, p=0.015) all were lower after dapagliflozin treatment compared to placebo (Table 2). Furthermore, and consistent with the lower plasma lactate levels during exercise, skeletal muscle lactate levels were lower after dapagliflozin treatment (-464.01 (-840.41, -87.6004) pmoles*mg tissue^{-1}, p=0.033, Table 3). Furthermore, the TCA cycle intermediate succinate (-15.2619 (-27.3731, -3.1507) pmoles*mg tissue^{-1}, p=0.0065, Table 3) was significantly lower after dapagliflozin treatment, while citrate (-14.4604 (-28.4890, -0.4317) pmoles*mg tissue^{-1}, p=0.060, Table 3) showed a trend towards lower levels after dapagliflozin treatment. An overview of all amino acids and organic compounds can be found in Table 2 and 3.

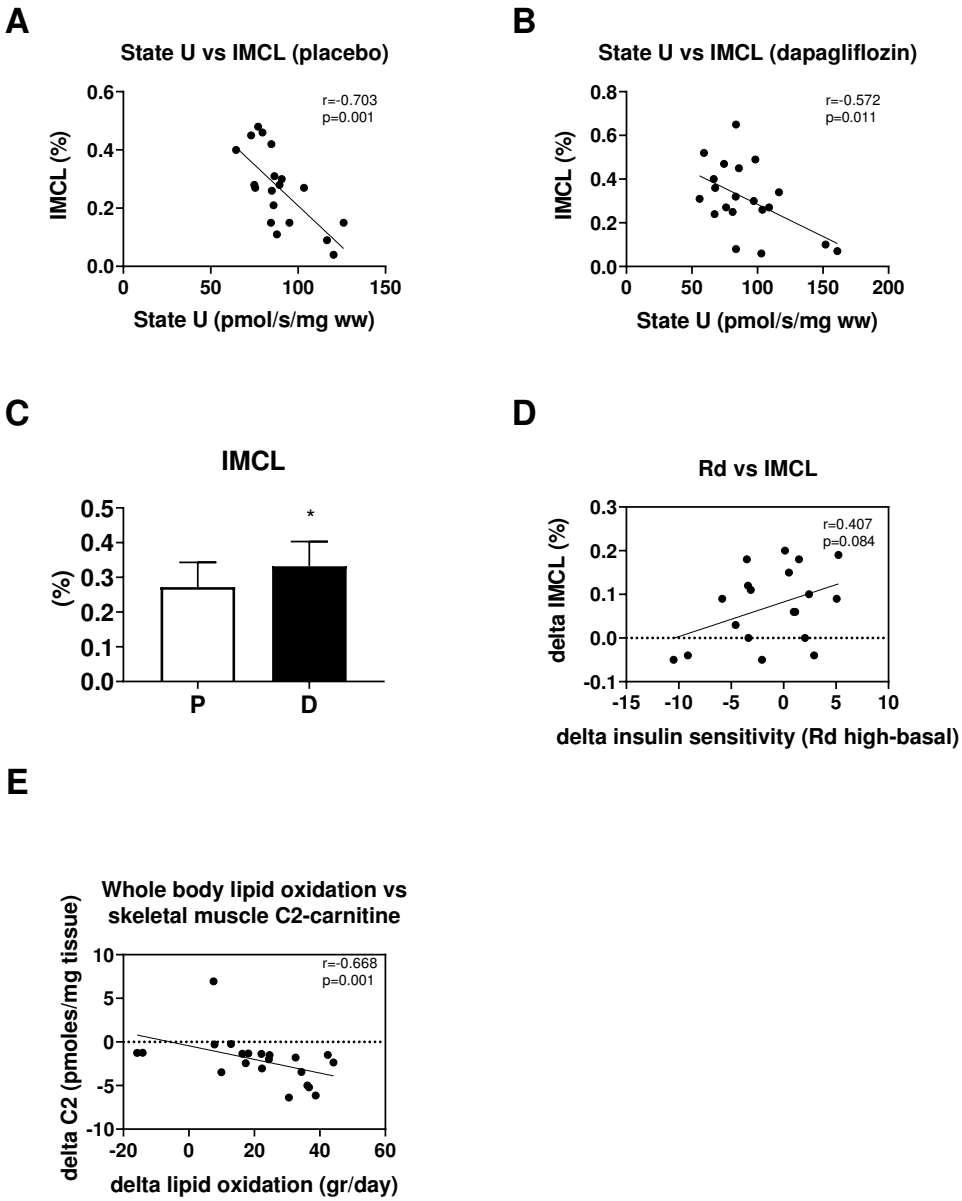

Figure 2. a) correlation of IMCL and State U mitochondrial respiration (maximal mitochondrial respiration with octanoylcarnitine addition) after placebo treatment, b) correlation of IMCL and State U mitochondrial respiration after dapagliflozin treatment, c) intramyocellular lipid (IMCL) content, d) correlation of change in insulin sensitivity (delta $R_{dhigh-basal}$) and change in IMCL, and e) correlation of change in whole body lipid oxidation and change in skeletal muscle C2 carnitine levels. Results (n=20) are in least squares mean (LSM) and 95% confidence interval (CI), obtained through a linear mixed model. Pearson correlations are performed using a linear regression model, except for figure 2e, where spearman correlation was performed. * P<0.05 is considered significantly different.

Table 2. Skeletal muscle levels of amino acids measured during the end-of-treatment visit. All parameters are in pmoles*mg tissue^{-1} and expressed in least square means and 95% CI. The p-value is obtained from a Wilcoxon signed rank test.

	Dapagliflozin		**Placebo**		**P-value**
	LSmean	**95% CI**	**LSmean**	**95% CI**	
Glycine	518.54	(455.17, 581.90)	593.87	(530.51, 657.24)	P=0.065
Alanine	1127.44	(994.13, 1260.74	1277.60	(1144.29, 1410.91)	P=0.013
Serine	1118.43	(690.56, 1546.29)	1471.98	(1044.11, 1899.84)	P=0.32
Proline	363.24	(311.21, 415.26)	409.27	(357.25, 461.30)	P=0.009
Valine	192.10	(175.44, 208.76)	206.70	(190.05, 223.36)	P=0.009
Leucine/Isoleucine	246.26	(225.60, 266.93)	259.45	(238.78, 280.12)	P=0.20
Methionine	39.50	(36.94, 42.06)	43.15	(40.58, 45.71)	P=0.051
Histidine	228.33	(204.10, 252.57)	237.36	(213.12, 261.59)	P=0.48
Phenylalanine	42.97	(38.86, 47.09)	45.80	(41.69, 49.92)	P=0.27
Tyrosine	47.76	(42.96, 52.56)	52.02	(47.22, 56.83)	P=0.11
Aspartic acid	185.67	(155.75, 215.59)	162.61	(132.69, 192.53)	P=0.060
Glutamic acid	1470.83	(1242.96, 1698.70)	1637.54	(1409.67, 1865.41)	P=0.015
Ornithine	102.80	(81.94, 123.67)	110.85	(89.98, 131.72)	P=0.15
Citrulline	25.50	(15.00, 36.01)	26.07	(15.57, 36.57)	P=0.58
Arginine	187.30	(142.96, 231.63)	213.10	(168.76, 257.43)	P=0.11

Table 3. Skeletal muscle levels of organic acids, Citrate Synthase and Creatine acetyltransferase activity, measured during the end-of-treatment visit. All parameters are in pmoles*mg tissue^{-1} and expressed in least square means and 95% CI. The p-value is obtained from a Wilcoxon signed rank test.

	Dapagliflozin		**Placebo**		**P-value**
	LSmean	**95% CI**	**LSmean**	**95% CI**	
Lactate	2550.49	(2170.08, 2930.90)	3014.49	(2634.08, 3394.90)	P=0.033
Pyruvate	89.02	(72.76, 105.28)	89.46	(73.20, 105.73)	P=0.99
Succinate	67.08	(56.14, 78.01)	82.34	(71.41, 93.27)	P=0.007
Fumarate	25.73	(22.96, 28.49)	26.05	(23.29, 28.82)	P=0.89
Malate	211.23	(189.07, 233.40)	213.64	(191.47, 235.81)	P=0.96
a-Kg	20.29	(15.30, 25.27)	25.80	(20.82, 30.79)	P=0.013
Citrate	106.33	(92.7386, 119.92)	120.79	(107.20, 134.38)	P=0.060
Citrate Synthase (µmol/min/gr tissue)	8.20	(6.81, 9.59)	8.57	(7.18, 9.96)	P=0.17
Carnitine Acetyltransferase (µmol/min/gr tissue)	4.61	(4.08, 5.14)	4.99	(4.46, 5.53)	P=0.076

DISCUSSION

We previously reported that 5-weeks of SGLT2 inhibitor treatment in patients with T2DM increased whole-body 24h fat oxidation, reduced IHL content and trunk fat mass (4). Furthermore, we reported a change in respiratory exchange ratio between day- and nighttime, indicative for an improvement of metabolic flexibility (4). No change in energy expenditure after dapagliflozin was found. Dapagliflozin treatment did improve hepatic and adipose tissue insulin sensitivity, but no change in whole body insulin sensitivity was reported (4). Here, we investigated the hypothesis that SGLT2 inhibition induces caloric restriction-like effects, such as alleviated lipid-induced mitochondrial changes in human skeletal muscle. We show that dapagliflozin had pronounced effects on skeletal muscle acylcarnitine and IMCL levels, as well was on amino acids and TCA cycle intermediates, but did not affect skeletal muscle mitochondrial function.

Dapagliflozin treatment had marked effects on skeletal muscle acylcarnitine species, including higher long-chain acylcarnitines and lower acetylcarnitine levels measured by metabolomics. It has been shown before that 12h fasting in obese participants did result in decreased acetylcarnitine levels (32) and together with higher long-chain acylcarnitines levels this may reflect an increase in skeletal muscle lipid oxidation upon prolonged fasting. Consistently, we here found that the change in C2-carnitine levels between treatments correlated negatively with the change in whole-body lipid oxidation. Levels of acetylcarnitine in skeletal muscle have been associated with insulin resistance, with low acetylcarnitine concentration in patients with T2DM, compared to healthy lean participants (17). It has been suggested that such low acetylcarnitine levels in type 2 diabetes patients reflect a reduced capacity to form acylcarnitines upon mitochondrial substrate overload (14, 33), and are paralleled by metabolic inflexibility. However, in the current study, dapagliflozin treatment increased lipid oxidation and increased the levels of long-chain acylcarnitine levels in skeletal muscle, reflecting high muscle fat oxidation flux, as has also been observed upon fasting in preclinical studies (34). Following increased β-hydroxybutyrate levels after dapagliflozin treatment in this study (4), also skeletal muscle C4-OH carnitine was increased. C4-OH carnitine has been linked to both fasting and ketosis. C4-OH can be produced from the ketone body D-(-)-3-hydroxybutyrate (D-3HB) (35), and it is known that ketone bodies are formed from fatty acid β-oxidation in the liver (36). Together, our results suggest that dapagliflozin increases the flux of lipid and ketone oxidation in skeletal muscle, effects similar as observed after calorie restriction and/or prolonged exercise training.

The increase in IMCL after dapagliflozin treatment seems paradoxical when considering the changes in muscle acetylcarnitine and other acylcarnitine species reflecting lipid oxidation rates. However, we and others have previously shown that interventions that improve skeletal muscle lipid oxidation, such as exercise training, resveratrol treatment, and prolonged fasting also increase the level of IMCL (10, 37, 38). Under such conditions, an increase in IMCL is not due to excessive lipid storage, but resembles a metabolic remodelling of skeletal muscle in which lipid droplets are stored in the close vicinity of mitochondria in order to fuel mitochondria under conditions of high energy demand (such as exercise) or low exogenous substrate supply (such as fasting). Consistently, the change in IMCL content tended to correlate positively with the change in insulin sensitivity between treatments, most likely reflecting that those volunteers that showed the most pronounced metabolic remodeling of skeletal muscle, also showed an increase in insulin sensitivity. Therefore, the increase in IMCL is consistent with the hypothesis that dapagliflozin exerts calorie-restriction like effects on skeletal muscle, leading to a more metabolic healthy skeletal muscle lipid phenotype. Such a phenotype involves lipid droplet dynamics, as we have recently shown that IMCL storage under healthy (for example endurance trained) conditions mainly involved the storage of lipids in small lipid droplets in type 1 muscle fibres and coated with proteins, such as PLIN5, whereas in the diabetic phenotype skeletal muscle lipids are mainly stored as large lipid droplets in type 2 muscle fibres (39). Future analysis of lipid droplet dynamics upon dapagliflozin treatment is needed to further investigate how such muscle metabolic remodeling may be involved in the beneficial health effects of dapagliflozin.

Next to an increased lipid oxidation, we here report lower levels of TCA cycle intermediates and lactate in skeletal muscle, which may be reflective of a lower glycolytic flux (32). Interestingly, levels of several glucogenic skeletal muscle amino acids (valine, proline, alanine and glutamic acid) decreased after dapagliflozin as well. Specifically glucogenic amino acids in skeletal muscle are reported to decrease after fasting or starvation, either as a result of increased efflux towards plasma in order to act as a substrate for hepatic gluconeogenesis (40), or to replenish TCA cycle intermediates (41), however the latter seems unlikely here given the observed decrease in TCA cycle intermediates. However, dapagliflozin is known to increase endogenous glucose production, mainly due to an increase in hepatic gluconeogenesis and the current findings therefore may suggest enhanced flux of amino acids from skeletal muscle towards gluconeogenesis (42). However, it should be noted that in our current study, no

sign of muscle protein breakdown or elevated nitrogen excretion was observed and we have suggested that the increase in EGP upon dapagliflozin in our study may be largely due to increased delivery of fatty acids and glycerol as substrates for gluconeogenesis (4). Consistently, also after prolonged fasting or starvation the amino acid release from muscle towards plasma decreases (43) and there is an apparent decrease in nitrogen excretion (41) and enhanced contribution of lipid metabolites to gluconeogenesis (44).

Despite the indications of enhanced skeletal muscle fat oxidation capacity following dapagliflozin treatment, mitochondrial function was unaltered after SGLT2 inhibition, both when measured *in vivo* or *ex vivo*. Consistently, calorie restriction in overweight to obese participants did not alter mitochondrial function or density (45). Moreover, we previously showed that prolonged fasting for 60 hours in healthy lean participants – if anything – slightly reduced mitochondrial function (46). These finding suggest that improvements in mitochondrial fat oxidative capacity and/or flux are not necessarily reflected in changes in (maximal) mitochondrial respiration. Improvements in the aforementioned lipid droplet dynamics and/or lipid droplet – mitochondria interaction could be involved in a more metabolic healthy skeletal muscle phenotype (39). Alternatively, the duration of the study may have been too short to elicit changes in mitochondrial respiratory capacity.

To summarize, dapagliflozin treatment for 5 weeks resulted in alterations in acylcarnitine species that reflect higher skeletal muscle lipid oxidation rates. Next to enhanced lipid oxidation, IMCL content increased as well suggesting that dapagliflozin leads to a metabolic remodeling of skeletal muscle more closely reflecting a healthy, metabolic flexible muscle phenotype. The long-term effects of such muscle adaptions for insulin sensitivity and metabolic health, as well as the investigation of similar effects on other metabolic tissues such as liver and heart needs future research.

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Declaration of interest

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Author contribution

P.S., V.S.H., M.H., J.H., E.P., B.H., J.O. and R.E. designed and conceived the study. Y.K., M.L., B.D. E.P., E.K., G.S., J.J., J.H., M.H., V.S.H., T.K., D.M., E.P. and P.S. designed and performed the experiments. Y.K., M.L., B.D., V.S.H., T.K., D.M., E.P., and P.S. analyzed the data. Y.K., P.S. and J.O. drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

Data sharing Data underlying the findings described in this manuscript may be available upon request in accordance with AstraZeneca's data sharing policy described at https://astrazenecagroup-dt.pharmacm.com/DT/Home

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Chapter 8

General discussion

Deposition of excessive fat, especially in ectopic tissues like liver and skeletal muscle, contributes to the development of metabolic disturbances and insulin resistance. Reducing of ectopic fat storage in patients with T2DM could therefore alleviate lipid-induced insulin resistance. Several classes of medication are known to have effects on skeletal muscle lipid metabolism, either by (indirectly) increasing lipid oxidation in skeletal muscle or through decreasing availability of fatty acids towards skeletal muscle. The aim of this PhD thesis is to identify mechanisms behind lipid-induced insulin resistance in skeletal muscle, and whether supplementation or a pharmacological approach can alter whole-body lipid metabolism and alleviate lipid-induced insulin resistance.

How does skeletal muscle lipid storage relate to insulin sensitivity?

Lipid accumulation inside skeletal muscle contributes to the development of insulin resistance (1). Both histochemical analysis and MRS measurements, have revealed that IMCL levels are negatively correlated with insulin sensitivity (2, 3). In **chapter 3**, we observed that sedentary obese insulin resistant participants have higher IMCL values in the m. vastus lateralis, compared to sedentary insulin sensitive participants, when measured by ^{1}H-MRS. To some extent, the level of IMCL contributes to the level of insulin resistance in sedentary people. However, this does not account to all humans, since the level of IMCL in endurance trained athletes are similar or sometimes even higher, compared to people with insulin resistance (3-5). We have also observed that the level of IMCL in the m. vastus lateralis of endurance trained athletes in chapter 3 is similar to insulin resistant participants, but the athletes are more insulin sensitive. This implies that insulin sensitivity is not affected by the level of IMCL in endurance trained athletes.

As explained in **chapter 2**, a possible mismatch between skeletal muscle fatty acid uptake and oxidative capacity, especially at the level of TCA flux, could determine the level of IMCL deposition. Reduced fat oxidative capacity decreases shuttling of long-chain acyl-CoA towards mitochondrial β-oxidation, but rather towards synthesis of lipid intermediates (6). Lipid intermediates, for example DAG species and ceramides, are increased in patients with T2DM and insulin resistant participants (7). Estimates of oxidative capacity can be made by measuring in *ex vivo* mitochondrial respiration. In **chapter 3**, we have reported differences in *ex vivo* mitochondrial respiration between insulin sensitive participant and insulin resistant participants. Although we have not

measured lipid-turnover in terms of tracer kinetics, a higher mitochondrial respiration and simultaneously lower IMCL content in insulin sensitive participant may imply a higher lipid-turnover, when compared to insulin resistant participants. The importance of a high mitochondrial oxidative capacity is also illustrated in **chapter 7**, where we report a strong negative correlation between IMCL content and maximum mitochondrial fat oxidative phosphorylation capacity. However, in the case of endurance trained athletes both *ex vivo* mitochondrial respiration and IMCL content is high, as was shown in **chapter 3**. It is known that athletes use IMCL for energy delivery during exercise and in combination with a high mitochondrial fat oxidative capacity, they can have a high lipid turnover. Previous data shows that endurance trained athletes who have a high mitochondrial oxidative capacity show less decrease in insulin sensitivity upon acute lipid infusion, compared obese participants (8). Simultaneously, IMCL levels after lipid infusion were unaffected in the athletes group, while the obese group showed an increase in IMCL (8). This shows that a high oxidative capacity might be one of the determinants of lipid-turnover (9). Also, mitochondrial respiration capacity in sedentary participants may determine the accumulation of skeletal muscle lipids and further development of insulin resistance.

Can supplements improve lipid metabolism and lipid accumulation in patients with T2DM?

Supplementation of essential compounds in skeletal muscle oxidative machinery, used for improving skeletal muscle oxidative capacity or fat metabolism, have shown promising results in rodent studies. For example, NAD^+ supplementation in rodents increased mitochondrial respiration (10), and resveratrol treatment in rodents was able to increase mitochondrial TCA cycle capacity and decrease IMCL deposition (11). Specifically for the use of NAD^+ precursors as supplements in patients with T2DM, human studies did not show the improvement of mitochondrial oxidative capacity found in rodent models, as we reviewed in **chapter 2**. Furthermore, IMCL levels remain unaffected as well. While insulin sensitivity does not alter as well, it is not likely that the composition of lipid intermediates, or the subcellular localization of lipids within skeletal muscle is altered. In **chapter 2**, multiple human trials have been reviewed that show an improved mitochondrial oxidative capacity after resveratrol treatment (12-14). Subsequently, those trials show either no change in IMCL, or an increased IMCL content (12, 13). Resveratrol supplementation is known to increase skeletal muscle lipid oxidation and shows calorie-restriction like effects (12). These adaptations are similar to exercise

training and prolonged fasting, with improved lipid oxidation and increased IMCL (15, 16). Therefore, there is some evidence that resveratrol has a minor increasing effect on oxidative capacity, without evidently improving insulin sensitivity. Although increasing oxidative capacity seems a promising approach for improving ectopic lipid accumulation, the effect of these supplements seems not to be strong enough to improve insulin sensitivity.

Carnitine supplementation helps to increase acetylcarnitine formation in participants with impaired glucose tolerance (17), and T2DM, as was shown in **chapter 5**. Although acetylcarnitine formation is correlated with degree of insulin sensitivity (18), it is still quite uncertain how acetylcarnitine formation can reduce insulin resistance, and whether this is a result of alleviating lipid accumulation in skeletal muscle. Carnitine is used in skeletal muscle to translocate long-chain acyl-CoAs from cytosol towards mitochondria, in order to facilitate fat oxidation (19, 20). Furthermore, by forming acylcarnitines, redundant acyl chains can be removed from the mitochondria, for example during (over) feeding (19-22), alleviating substrate competition on the level of mitochondria (20, 23). An improvement of metabolic flexibility has been reported after carnitine supplementation in participants with impaired glucose tolerance, suggesting that improved acetylcarnitine formation indeed alleviates substrate competition in mitochondria (17). In **chapter 4**, a study with acute lipid infusion in healthy young males, as a model of insulin resistance, was performed to study the effect of carnitine infusion on metabolic flexibility and insulin sensitivity. As expected, during a two-step euglycemic hyperinsulinemic clamp, lipid infusion increased free fatty acid levels in plasma and induced metabolic inflexibility and insulin resistance. However, lipid infusion and additional carnitine infusion did not alleviate metabolic inflexibility and was also unable to improve insulin resistance. During insulin + lipid infusion, we found higher lipid oxidation and higher plasma acetylcarnitine levels, assumed to reflect an increased efflux of β-oxidation intermediates by tissues such as liver and muscle (6, 24). Adding carnitine infusion with lipid infusion, resulted in an even higher increase in plasma acylcarnitines, suggesting that free carnitine availability is necessary for efflux of β-oxidation intermediates from the liver. Despite the elevated plasma acylcarnitines, skeletal muscle carnitine availability and formation of acetylcarnitine was unchanged after lipid infusion and carnitine infusion, compared to lipid infusion and placebo. Carnitine uptake into skeletal muscle is insulin-mediated via the sodium dependent organic cation transporter (OCTN2) (25). The acute insulin resistance, induced by lipid

infusion, potentially hampered the insulin-mediated uptake of carnitine into skeletal muscle. Although carnitine did not improve acetylcarnitine and acylcarnitine formation in skeletal muscle, this trial shows the importance of increasing free carnitine availability for the efflux of β-oxidation intermediates.

In **chapter 5**, oral carnitine supplementation was administered for 3 months in patients with T2DM. We hypothesized that carnitine supplementation was able to improve insulin sensitivity, as a result of increased capacity to form acetylcarnitine. Oral carnitine supplementation did increase acetylcarnitine formation in skeletal muscle as measured by ^{1}H-MRS and we found increased insulin sensitivity after 3 months of carnitine supplementation, mainly caused by an increase of NOGD. This implies that the skeletal muscle uptake of glucose is less hampered by insulin resistance, while glucose oxidation not necessarily improves with carnitine supplementation. Metabolic flexibility as measured by the delta of RER between high insulin phase and basal, is not changed with carnitine supplementation. This would mean that the increase in carnitine availability through carnitine supplementation, does not necessarily alleviate mitochondrial substrate competition in patients with T2DM. However, since insulin sensitivity is improved in skeletal muscle, increased acylcarnitine formation and translocation of acylcarnitines towards mitochondria, could prevent the accumulation of acyl-CoA's and lipid intermediates in cytosol. Besides increased skeletal muscle insulin sensitivity, insulin-induced suppression of endogenous glucose production was higher after carnitine supplementation. This coincides the trend in reduced intrahepatic lipid content reported in this trial, after carnitine supplementation. This would suggest, that increasing free carnitine availability increases the efflux of β-oxidation intermediates, thus decreasing intrahepatic lipid content.

Carnitine supplementation is effective in increasing free carnitine availability, as reported in a study of Bruls et al. (17), but also reported in **chapter 4**, with an increase in plasma free carnitine after carnitine supplementation. The increased formation of acylcarnitines with carnitine supplementation is therefore effective in improving insulin sensitivity, most likely as a result of lower lipid accumulation and more complete β-oxidation, in both liver and skeletal muscle. Measuring acylcarnitine species and IMCL content in skeletal muscle of these patients could reveal what the impact of carnitine supplementation is on preventing accumulation of IMCL and lipid intermediates.

Can a pharmacological approach improve lipid metabolism and lipid accumulation in patients with T2DM?

The classes of medication, elaborated in **chapter 2**, all have been reported to result in changes in whole-body lipid metabolism, but exert their effects in different tissues. For example fibrates (PPARα), PPARγ, acipimox and statins are compounds that decrease plasma TG and FFA levels, either through increased lipid oxidation and decreased TG synthesis in the liver (fibrates) (26, 27), higher TG clearance by adipose tissue (PPARγ) (28), decreasing cholesterol synthesis in the liver (statins) (29), or inhibition of lipolysis in adipose tissue (acipimox) (30). Since elevated plasma FFA levels are correlated with the development of insulin resistance in skeletal muscle (31), which we used as a model to induced insulin resistance in **chapter 4**, lowering of plasma FFA levels is a way to alleviate insulin resistance. Overall, the effect of each of these compounds is associated with an improvement of insulin sensitivity, except for statins.

Acute acipimox treatment is very potent in decreasing IMCL and subsequently increasing insulin sensitivity (30, 32), although the rebound effect makes it a less suitable candidate for long-term treatment to alleviate lipid-induced insulin resistance. Although the number of human trials using fibrates and PPARγ as a treatment to improve insulin sensitivity and IMCL is limited, there is evidence that these compounds not only lower FA availability towards skeletal muscle but might stimulate PPAR related genes in skeletal muscle as well. Trials report no evident change in IMCL, but fractional synthesis rates of TG in skeletal muscle increased with fibrates (33) and basal lipid oxidation increased by PPARγ treatment (34). This may imply that lipid-turnover in skeletal muscle could be increased as a result of fibrate and PPARγ treatment. A compound that hypothetically can increase oxidative capacity and with this, lipid turnover, are PPARδ agonists. Skeletal muscle have a high expression of PPARδ (35). Increased lipid oxidation rate, increased mitochondrial biogenesis and reduced lipid accumulation or lowered accumulation of lipid intermediates can be expected (35-38), as reported in rodent models. Human trials with PPARδ agonists are not performed as of today.

Metformin has a glucose-lowering effect, by direct and indirect activation of AMPK in liver, via inhibition of complex I in mitochondria (39). In the liver, AMPK is suggested to stimulate lipid oxidation and to inhibit both lipid synthesis (40) and gluconeogenesis (41). Eventually, two trials report an increased insulin sensitivity after metformin treatment, while IMCL levels remained unchanged or decrease. The number of trials

with metformin treatment, measuring both IMCL levels or lipid turnover, in combination of measuring insulin sensitivity, is still scarce. The insulin sensitivity increasing effect of metformin can potentially be caused by a number of effects, for example less glucotoxicity, increasing lipid-turnover by skeletal muscle AMPK activation, or less FA availability towards skeletal muscle. It is likely that a combination of the aforementioned effects play a role in metformin treatment, resulting in a more metabolically healthy skeletal muscle.

In **chapter 6**, we report a double-blinded randomized, placebo-controlled, cross-over study with 5 weeks of dapagliflozin treatment. Sodium-glucose cotransporter 2 inhibitors (SGLT2i) inhibit glucose and sodium reabsorption in the proximal renal tubules, with about 50-100g glucose loss per day in urine. This glucose loss in urine is a form of mild caloric restriction and the decrease in plasma glucose levels (42) has been shown to increase glucagon levels (43) and decrease insulin secretion (44). More important, fasting fatty acid oxidation increases after SGLT2i (45), and body weight (46) and intrahepatic lipid content (47) is reduced after SGLT2i treatment, suggesting a decrease in (ectopic) lipid accumulation. Several trials have reported an increased skeletal muscle insulin sensitivity after SGLT2i (45, 48, 49), suggesting alleviated lipid-induced insulin resistance as a result of higher fatty acid oxidation. Dapagliflozin treatment in our trial effectively increased lipolysis, as reflected in increased plasma FFA levels. Dapagliflozin decreased 24hr energy balance, mostly caused by a negative lipid balance. Both hepatic and adipose tissue insulin sensitivity, as measured by a two-step euglycemic hyperinsulinemic clamp, increased with dapagliflozin treatment. The increase in hepatic insulin sensitivity coincides with a decrease in intrahepatic lipid content. The change in respiratory exchange ratio between day and night, as a measure of metabolic flexibility, increased with dapagliflozin. However, skeletal muscle insulin sensitivity remained unaffected with dapagliflozin, although fat oxidation was significantly increased during insulin infusion. No improvement in peripheral insulin sensitivity was also observed by Latva-Rasku et al. (47), whereas other studies with SGLT2 inhibitors reported improvements in peripheral insulin sensitivity (45, 49, 50). Possibly the difference in baseline glucose control and different rates of insulin infusion could explain the discrepant findings here, compared to other trials. Another, more physiological explanation could be the so-called post-marathon paradox, where insulin sensitivity is decreased rather than increased after exercise, which is explained by increased plasma FFA levels as a result of exercise. Comparably, also in our trial, we found increased

plasma FFA levels as a result of higher lipolysis upon SGLT2i (51). These fatty acid levels were still increased at the time of the measurement of insulin sensitivity, so this SGLT2i-induced increase in plasma FFA levels may have blunted a potential treatment effect on insulin sensitivity. However, more research is needed to investigate this hypothesis. Regardless of the effect of SGLT2i on insulin sensitivity, this trial indicates that SGLT2i induces a negative energy balance and calorie-restriction like effects, resulting in marked improvement of whole-body metabolic health.

Calorie restriction like effects can improve skeletal muscle fat oxidative and mitochondrial capacity (52). Since insulin resistance in skeletal muscle is associated with reduced mitochondrial function (15, 53, 54), alleviating whole-body and mitochondrial lipid oxidation is important in patients with T2DM. Preventing mitochondrial substrate oversupply, by converting excessive mitochondrial acetyl-CoA towards acetylcarnitine (55), could improve metabolic flexibility in patient with T2DM. Therefore, in **chapter 7**, we investigated the hypothesis that SGLT2 inhibition induces calorie restriction-like effects, and subsequently alleviates lipid-induced mitochondrial dysfunction and substrate competition on mitochondrial level. Both *in vivo* mitochondrial function, measured as PCr recovery with ^{31}P-MRS, and *ex vivo* mitochondrial function remained unaffected with dapagliflozin treatment. In contrast to the decreased trunk fat mass and hepatic lipid content reported in **chapter 6**, we found increased IMCL content after dapagliflozin treatment. IMCL content after both placebo and dapagliflozin, correlates negatively with maximum mitochondrial fat oxidative phosphorylation capacity. A low mitochondrial oxidative capacity is suggestive to be the driving force in IMCL accumulation. However, the change in IMCL content upon SGLT2i did show a trend to a positive correlation with the changes in skeletal muscle insulin sensitivity. Other calorie-restriction like interventions, as exercise training and prolonged fasting, have also reported an increased IMCL (15, 16). The increased whole-body and skeletal muscle fat oxidation, as reported in **chapter 6**, and increased IMCL content after dapagliflozin treatment, suggests a higher lipid-turnover independent of an increase in mitochondrial respiration capacity. Higher skeletal muscle acylcarnitine levels and lower glycogenic amino acid and TCA cycle intermediates found in this study, reflect the higher skeletal muscle lipid oxidation and lower carbohydrate oxidation after dapagliflozin. Eventually, 5 weeks of SGLT2i, results in whole-body and skeletal muscle calorie-restriction like effects and increase lipid oxidation rates on a whole-body and skeletal muscle level, without an improvement in skeletal muscle insulin sensitivity.

Future perspectives

As reviewed in **chapter 3**, total IMCL content is not correlated with insulin resistance. This was also the conclusion of **chapter 7**, where the increase in IMCL was accompanied by an increase in fat oxidation and resembled adaptations towards a metabolic healthy skeletal muscle. While exercise and prolonged fasting are capable of alleviating insulin resistance (8, 56), the calorie restriction like effects of SGLT2i after short-term treatment did not increase insulin sensitivity in patients with T2DM. Long-term treatment with SGLT2i could reveal if insulin sensitivity in skeletal muscle does improve in time, as a result of the increased lipid oxidation rates.

Chapter 7 revealed a decrease in skeletal muscle acetylcarnitine, but mostly an increase in medium- and long-chain acylcarnitines, without increasing free carnitine availability. As discussed, these results reflect a higher lipid oxidation in skeletal muscle. A previous study with carnitine supplementation has reported an increase in short-, medium- and long-chain acylcarnitines in skeletal muscle (17). With SGLT2i treatment increasing whole body lipid oxidation and carnitine supplementation increasing acylcarnitine formation and efflux of acylcarnitines out of liver and skeletal muscle, a combined treatment of SGLT2i and carnitine may have a synergistic effect on lipid turnover and insulin sensitivity.

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Addendum

SAMENVATTING

Een wereldwijde toename van mensen met overgewicht of (morbide) obesitas blijft een probleem voor de volksgezondheid in de huidige samenleving (1). Een onbalans tussen inname van calorierijke maaltijden en lage fysieke activiteit, zorgt voor de ontwikkeling van obesitas en daarmee een toename van verschillende chronische ziekten zoals hart- en vaatziekten, leververvetting en type 2 diabetes (2, 3). De opslag van overtollig vet bij mensen met obesitas, voornamelijk in ectopische weefsels als lever en skeletspier, draagt bij aan de ontwikkeling van metabole stoornissen en insuline resistentie (4, 5). Potentieel kan het verminderen van de opslag van ectopisch vet bij patiënten met type 2 diabetes, zorgen voor een vermindering van insuline resistentie. Van verschillende typen medicijnen weten we dat ze effect hebben op het vet metabolisme in de (skelet) spier. Dit kan door het verhogen van vetoxidatie in de spier, of door een afnemende beschikbaarheid van vetzuren naar de spier toe.

Het doel van dit proefschrift is het identificeren van mechanismen achter de ontwikkeling van insuline resistentie door de opslag van ectopisch vet in skeletspier. Daarnaast is er gekeken naar de mogelijkheid om via supplementen of een farmacologische benadering de omzetting van lipiden in het lichaam te veranderen, en daarmee de insuline resistentie te verminderen.

Hoofdstuk 2 brengt met behulp van de beschikbare literatuur in kaart wat het effect van verschillende soorten supplementen, nodig voor suiker en vet metabolisme in skeletspier, en verschillende soorten medicatie gebruikt voor type 2 diabetes en hyperlipidemie en hypercholesterolemie, op de lipidenomzet en vetopslag in de spier is. Daarnaast werd in dit hoofdstuk ook gekeken of een aanpassing van de lipidenomzet en vetopslag, ook leidt tot een verbetering in insuline gevoeligheid in de spier. Binnen de groep aan supplementen kunnen we de volgende conclusies trekken: 1) supplementatie van NAD^+ precursoren lijkt de mitochondriële oxidatieve capaciteit onvoldoende te verhogen, met als gevolg dat er geen effect wordt gezien op vetopslag en insuline gevoeligheid in de spier; 2) resveratrol supplementatie laat een verbeterde capaciteit voor vetoxidatie zien, vergelijkbaar aan interventies met inspanning en langdurig vasten, met als gevolg een verhoging van vetopslag in de spier zonder verbetering van insuline gevoeligheid; 3) supplementatie van carnitine verbetert de β-oxidatie en verhoogt de metabole flexibiliteit, zonder een duidelijke verbetering in insuline gevoeligheid. Binnen de groep van medicatie kunnen we de volgende conclusie trekken: 1) PPARδ agonisten,

die uitsluitend mitochondriale biogenese stimuleren in skelet spier, lijken onvoldoende in staat te zijn om vetopslag of lipidenomzet te verbeteren, zonder effect op insuline gevoeligheid; 2) PPARα agonisten, GLP-1 agonisten en DPPIV-inhibitoren kunnen via een verhoogde vetoxidatie in skelet spier en verlaging van vetzuur afgifte vanuit de lever, de lipidenomzet in de spier verbeteren, met een verbetering in insuline gevoeligheid tot gevolg; 3) PPARγ agonisten en het acute effect van acipimox, leiden tot sterke plasma vetzuur daling, door het remmen van lipolyse en verhoogde vetzuuropname in vetweefsel, met als gevolg een sterke verbetering in insuline gevoeligheid in de spier door lagere vetopslag in de spier. Voornamelijk het verlagen van plasma vetzuur waarden, met als gevolg lagere vetopslag in de spier, lijkt een sterke verlaging van insuline resistentie te veroorzaken.

Met de kennis dat vetopslag in de spier bijdraagt aan de ontwikkeling van insuline resistentie, hebben we in **hoofdstuk 3** onderzocht hoe de hoge vetopslag in insuline gevoelige duuratleten verschilt van de vetopslag in obese insuline resistente mensen. In een cross-sectioneel onderzoek met 103 proefpersonen tonen we middels ^{1}H-MRS aan dat de vetopslag in m. vastus lateralis even hoog is in duuratleten en obese insuline resistente proefpersonen, en daarmee hoger dan insuline gevoelige sedentaire proefpersonen. Daarentegen is de insuline gevoeligheid, voornamelijk door een stijging in niet-oxidatieve glucose opname, hoger in duuratleten vergeleken met obese insuline resistente proefpersonen. De vetopslag in atleten bevindt zich voornamelijk rondom de mitochondriën, terwijl de vetopslag bij insuline gevoelige en resistente sedentaire proefpersonen minder rondom de mitochondriën is gepositioneerd. Verder is de mitochondriële respiratoire capaciteit het hoogst in duuratleten en het laagst in insuline resistente sedentaire proefpersonen. Dit kan suggereren dat de lipidenomzet in atleten hoger is dan in sedentaire proefpersonen, en de opslag van vet meer rondom de mitochondriën plaats vind. Eerder onderzoek heeft uitgewezen dat vet in atleten voornamelijk in oxidatieve type I spiervezels is opgeslagen in de intermyofibrillaire regio's, terwijl bij insuline resistente proefpersonen het vet voornamelijk in type II spiervezels is opgeslagen onder het sarcolemma, overeenkomend met de resultaten in **hoofdstuk 3**. Verder is bekend uit literatuur, dat voornamelijk schadelijke lipide-intermediairen zorgen voor een verminderde insuline signaleringsfunctie in skeletspier. Mogelijk dat de plek van vetopslag in de spier zorgt voor de verschillen die we zien in insuline gevoeligheid en glucose opname in skeletspier.

Aangezien uit literatuur blijkt dat het verhogen van carnitine beschikbaarheid via carnitine supplementatie effectief is in het verbeteren van vetoxidatie in skeletspier, en daarmee de metabole flexibiliteit verbeterd, blijft de vraag of carnitine supplementatie in staat is om de vetopslag geïnduceerde insuline resistentie kan verminderen of voorkomen. Daarom is het effect van carnitine supplementatie op insuline gevoeligheid en metabole flexibiliteit onderzocht in 8 jonge gezonde insuline gevoelige mannen. In **hoofdstuk 4** beschrijven we een placebo-gecontroleerd onderzoek, waarbij we een lipide-infusie gebruiken als model om de proefpersonen insuline resistent te maken. De infusie van lipiden verhoogt de plasma vetzuur concentratie en daarmee verhoogt het zowel insuline resistentie als metabole inflexibiliteit. Verder is er een verhoging van acylcarnitines in het plasma te zien, mogelijk als gevolg van verhoogde afgifte van tussenproducten van β-oxidatie door spierweefsel en lever. Gelijktijdige infusie van carnitine is echter niet in staat om de ontwikkelde insuline resistentie en metabole flexibiliteit te verminderen. Ondanks een verhoging van plasma acylcarnitine waarden ten opzichte van alleen lipide-infusie, is er geen verhoogde beschikbaarheid van carnitine te vinden in skeletspier. Mogelijk dat de acuut geïnduceerde insuline resistentie door lipide-infusie, voorkomt dat carnitine kan worden opgenomen in de skeletspier.

Omdat het effect van carnitine infusie in acuut geïnduceerde insuline resistentie teniet wordt gedaan door lipide-infusie, hebben we in **hoofdstuk 5** gekozen voor het rekruteren van patiënten met type 2 diabetes. We hebben onderzocht of 3 maanden carnitine supplementatie in 32 patiënten met type 2 diabetes, de carnitine beschikbaarheid en vorming van acetylcarnitine kan verhogen en daarmee de insuline gevoeligheid verbeterd kan worden. Met behulp van ^{1}H-MRS hebben we aangetoond dat de vorming van acetylcarnitine inderdaad verbeterd na carnitine supplementatie en dat insuline gevoeligheid in skeletspier verbeterd, voornamelijk door een stijging van niet-oxidatieve glucose opname in de spier. Gelijktijdig is er geen verbetering in metabole flexibiliteit opgemerkt, waarmee geïmpliceerd kan worden dat de verbeterde carnitine beschikbaarheid niet zozeer de afstemming van vet en koolhydraatverbranding in de mitochondriën veranderd, maar wel de glucose opname in de spier verhoogd. Mogelijk dat de supplementatie van carnitine de accumulatie van acyl-CoA's en lipide-tussenproducten in de skeletspier verlaagt. Verder rapporteren we een verbeterde onderdrukking van endogene glucoseproductie in de lever en een trend naar een verlaagde vetopslag in de lever, na carnitine supplementatie. Dit zou suggereren dat carnitine de afgifte van tussenproducten van β-oxidatie verhoogt, met als gevolg een

lagere intrahepatische vetopslag.

In **hoofdstuk 6** rapporteren we een dubbelblinde, gerandomiseerd, placebo gecontroleerde, cross-over studie met 26 patiënten met type 2 diabetes, welke gedurende 5 weken SGLT2i behandeling kregen. SGLT2i behandeling resulteert in een toename van glucose excretie in de urine, en zorgt daarmee voor een dagelijkse milde calorie restrictie. Ook is er reeds aangetoond dat deze vorm van calorie restrictie zorgt voor een daling van insuline secretie en toename van glucagon productie, en neemt de vetoxidatie toe. Deze metabole veranderingen lijken op andere vormen van calorie restrictie, zoals lang vasten en sporten. De hypothese is dat de toename in vetoxidatie, als gevolg van calorie restrictie, zorgt voor een afname van ectopisch vetopslag in de lever en spieren. De behandeling met dapagliflozine zorgde voor een verhoogde lipolyse en vetoxidatie, wat resulteerde in hogere plasma vetzuur waarden. Dapagliflozine zorgde ook voor een negatieve 24-uurs energiebalans, voornamelijk door een negatieve vetbalans. Verder verbeterde de insuline gevoeligheid in vetweefsel en in de lever. De verbetering in insuline gevoeligheid in de lever valt samen met een afname in intrahepatische vetopslag. Daarnaast leek de toename in vetoxidatie gedurende de nacht meer toe te nemen na het gebruik van dapagliflozine, wat een teken is voor een verbeterde metabole flexibiliteit. Deze effecten laten een duidelijke verbetering van de metabole gezondheid zien. De insuline gevoeligheid in de skeletspier is niet veranderd met dapagliflozine, in tegenstelling tot eerdere onderzoeken. Het uitblijven van een verbeterde insuline gevoeligheid in de spier kan veroorzaakt worden door de hogere plasma vetzuurwaarden die we zien na dapagliflozine behandeling, als gevolg van hogere lipolyse.

Interventies waarbij calorie restrictie optreedt, heeft in eerdere onderzoeken laten zien dat dit zowel vetoxidatie als mitochondriële capaciteit kan verhogen. Insuline resistente skeletspieren worden geassocieerd met een verminderde mitochondriële functie, als gevolg van een overmaat aan mitochondrieel substraat. Een van de mechanismen waarop het lichaam kan voorkomen dat deze substraten te veel gaan stapelen, is door de omzetting van acetyl-CoA naar acetylcarnitine, een mechanisme dat bij patiënten met type 2 diabetes niet optimaal functioneert. De behandeling met dapagliflozine in **hoofdstuk 6** heeft laten zien dat er calorie restrictie-achtige effecten ontstaan, in combinatie met een toename van vetoxidatie in skeletspieren. Daarom hebben we in **hoofdstuk 7** de hypothese onderzocht dat SGLT2i behandeling, via calorie restrictie-achtige effecten, de substraatcompetitie op mitochondrieel niveau verminderd.

In vivo en *ex vivo* mitochondriële respiratie blijven onveranderd na behandeling met dapagliflozine. Daarentegen is de opslag van intramyocellulaire lipiden na behandeling met dapagliflozine hoger dan na behandeling met placebo. Verder zien we ook dat de maximale oxidatieve fosforyleringscapaciteit negatief correleert met de opslag van intramyocellulaire lipiden, wat suggereert dat in patiënten met type 2 diabetes een lage mitochondriële oxidatieve capaciteit de drijvende kracht achter vetstapeling in de spier is. Verder zien we dat de verandering in insuline gevoeligheid, juist positief correleert met de verandering in vetopslag in de spier. De hogere vetoxidatie na behandeling met dapagliflozine en de hogere vetopslag in de spier zijn suggestief voor een hogere lipidenomzet, onafhankelijk van de mitochondriële respiratie capaciteit. De hogere vetoxidatie is ook terug te zien in de hogere acylcarnitine spiegels in de skeletspier. Deze resultaten laten zien dat 5 weken behandeling met een SGLT2i zorgt voor calorie restrictie-achtige effecten in het lichaam en specifiek ook in de skeletspier, waarmee de vetoxidatie en vetopslag wordt verhoogd, wat tekenend is voor een metabool flexibel spier fenotype. Analyse van dit fenotype, bijvoorbeeld het bepalen van vetdruppel dynamiek en de vetverdeling over verschillende spiervezel types, kan mogelijk meer informatie geven over de verandering in vetopslag in de spier. Behandeling van SGLT2i over een langere periode is nodig om de lange termijn effecten op insuline gevoeligheid en metabole gezondheid te bepalen.

CONCLUSIE

Het doel van dit proefschrift was het identificeren van mechanismen achter de insuline resistentie in skeletspier. Daarnaast is er ook gekeken naar de mogelijkheid om via supplementen of een farmacologische benadering de omzetting van lipiden in het lichaam te veranderen, en daarmee de insuline resistentie te verminderen. We kunnen concluderen dat vetopslag rondom de mitochondriën voordelig is, omdat dit geen insuline resistentie lijkt te induceren. Mogelijk is dit een van de tekenen van een hogere omzet van lipiden in de spier. Het stimuleren van een (relatief) hogere omzet van lipiden in de spier lijkt een positief effect te hebben op de verbetering van insuline resistentie in de spier. Dit kan mogelijk bewerkstelligd worden door een lagere opslag van (schadelijke) vetten in de skeletspier (carnitine supplementatie). Anderzijds kan ook de vetverbranding gestimuleerd worden (SGLT2i behandeling), wat een verbetering van metabole gezondheid en verhoging van insuline gevoeligheid in verscheidene weefsels geeft, behoudens de skeletspier.

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IMPACT PARAGRAPH

What is the main purpose of the research described in this thesis and what are the main results and conclusions?

Prevalence of type 2 diabetes is increasing rapidly not only in the Netherlands but worldwide. Obesity is an important underlying factor in the development of type 2 diabetes. Especially the storage of lipids in tissues other than fat tissue, for example muscle, liver, heart and pancreas is crucial. This increased prevalence is accompanied with co-morbidities in type 2 diabetes patients, reducing the life expectancy but also the quality of life. Furthermore, health care costs related to this are immensely high causing a severe economic-burden. This highlights the importance of understanding the aetiology of type 2 diabetes and developing effective treatment strategies to lower this economic but also social burden caused by this disease.

In **chapter 2**, a literature study was performed investigating the effect of supplementation and pharmacological agents on whole body lipid metabolism, and the effect on lipid storage and insulin sensitivity in muscle tissue. Current pharmacological treatments are focussed on lowering of glucose levels. However, it is necessary to reduce or prevent the storage of ectopic fat, which is one of the underlying problems that cause the development of type 2 diabetes. We conclude from this review that especially supplements and pharmacological agents that reduce the uptake of fat into muscle tissue, resulting in less fat in muscle tissue, are effective in increasing insulin sensitivity in muscle tissue. In addition, some pharmacological agents increase insulin sensitivity as a result of higher fat oxidation, without lowering the amount of fat in muscle tissue, which stresses that perhaps not the ectopic fat storage per se but the turnover of fat in muscle tissue is more important.

In **chapter 3** we further investigated the relation between fat storage in skeletal muscle and insulin sensitivity. To this end, we investigated a) trained, b) insulin resistant untrained, and c) insulin sensitive untrained individuals. Interestingly, a paradox was present with athletes and insulin resistance individuals both having high lipid storage in muscle with athletes having a high fat turnover and insulin resistant individuals a low fat turnover. Interestingly, the location of the lipids stored is different and apparently crucial in developing insulin resistance and type 2 diabetes. Athletes have a pronounced storage of fat connected to the mitochondria, where insulin resistant individuals store fat away from mitochondria, possibly underneath the skeletal muscle cell membrane, hampering

insulin signalling.

This challenged us to improve lipid oxidation/turnover by the use of supplements. In specify, we first investigated the potential effects of carnitine supplementation. Carnitine is well now for its role in lipid oxidation, but is more recently also pointed out as important player in maintaining proper glucose homeostasis. In **chapter 4** and **chapter 5**, we investigated the potential effect of carnitine to rescue lipid-induced insulin resistance. Since carnitine supplementation showed beneficial and promising effects in pre-diabetics, we here investigated the therapeutic potential in type 2 diabetes patients. We revealed that insulin resistance reduced, less lipids were stored in the liver and glucose levels tended to reduced. Therefore, we concluded that carnitine supplementation might be a strategy to treat type 2 diabetes and lower insulin resistance.

Next, in **chapter 6** and **chapter 7**, we investigated the potential of the pharmacological component SGLT2 inhibitors, a relatively new drug for patients with type 2 diabetes. SGLT2 inhibitors have been shown to improve glucose control and reduce body weight, as a result of glucose loss in urine. Other studies have shown that treatment with SGLT2 inhibitors show effects that are similar to the effects of exercise and following a diet. We investigated if SGLT2 inhibitor treatment results in less fat storage in liver and muscle and reduce insulin resistance. We showed that SGLT2 inhibitor treatment increases whole-body fat oxidation, decreases fat storage in liver and improves muscle metabolic health. Therefore, we concluded that SGLT2 inhibitor treatment can be used to lower whole-body storage of fat and to improve the health of muscle tissue.

What is the contribution of the results in this research to the scientific community and societal challenges?

The results of the studies described in this thesis contribute to the existing knowledge about the pathology and treatment of type 2 diabetes. With the knowledge obtained in this thesis, the understanding of the pathology and treatment of type 2 diabetes has increased. This provides opportunities for other researchers to develop new study ideas to further unravel the pathology of type 2 diabetes and improve the available treatment strategies.

With the rising prevalence of type 2 diabetes, effective treatment strategies for type 2

diabetes patients are increasingly important. Type 2 diabetes is associated with multiple comorbidities such as cardiovascular disease, liver disease and neuropathy. This has a major negative influence on the life expectancy but also reduces the quality of life of the patient. Therefore, the social impact of type 2 diabetes is high and highlights the importance of good treatment strategies. The results obtained in this thesis could contribute to this development of new treatment options and treatment strategies. In addition, the economic impact of type 2 diabetes is enormous due to very high healthcare costs to treat type 2 diabetes and occurring comorbidities. The results in this thesis can improve treatment and prevent/delay the onset of comorbidities and thereby reduce health care costs.

For whom are the research results interesting and relevant?
The results in the theses are interesting for different groups of people like researchers, medical professionals, pharmacological and insurance companies, as well as to the general society.

In the current thesis, more knowledge has been acquired on the role of muscle lipid accumulation and lipid turnover in insulin resistance and type 2 diabetes. Furthermore, supplementation and pharmacological treatment strategies focusing on improving muscle lipid storage and insulin resistance in type 2 diabetes revealed very promising effects to combat and treat type 2 diabetes. This knowledge is of great importance to researchers because it contributes to better understanding of the etiology of type 2 diabetes as well as the development of better treatment strategies for this chronic disease. Future studies can be developed based on this knowledge as discussed in the general discussion (**chapter 8**).

Furthermore, general practitioners, endocrinologist, dieticians and other health care professionals involved in treating type 2 diabetes patients could use the findings in this thesis to treat type 2 diabetes patients. SGLT2 inhibitors are new pharmacological compounds for the treatment of diabetes and the results of this thesis support the advantages to prescribe this compound in treating type 2 diabetes. Health care professionals are in direct contact with the patients and can highlight the advantage of using SGLT2 inhibitors. Furthermore, health care professionals can inform patients on the advantage of using carnitine supplementation on improving type 2 diabetes. Since carnitine supplements are available without prescription of physicians, this is a feasibly

strategy to treat diabetes.

Next, the results of this thesis are of interest to all individuals in our society especially individuals at risk or diagnosed with type 2 diabetes. The results of these studies can help individuals understand the consequences of obesity and whole-body fat storage on metabolic health and stimulate patients with type 2 diabetes to improve their lifestyle, either with or without the use of medication.

How can these target groups be involved in and informed about the research results, so that the acquired knowledge can be used in the future?
The results presented in this thesis are or will be published as original scientific articles in international well-recognized peer-reviewed journals. Hereby, the scientific articles will be available online and become assessable worldwide for scientist, medical doctors and other people interested. Additionally, the conducted research and obtained results in this thesis have been communicated to the scientific and medical community worldwide. Results were presented on multiple national (Annual Dutch Diabetes Research Meeting and the International Society for Magnetic Resonance in Medicine division Benelux) and international conferences (International Society for Magnetic Resonance in Medicine, European Association for the Study of Diabetes, Cell Symposium: Exercise Metabolism and on the Keystone Symposia) through oral presentations and posters. In this way, the obtained knowledge in this thesis was dissimulated, thereby contributing to new research ideas for future research as well as treatment therapies for type 2 diabetes.

In addition to presenting these results at (inter)national conferences, all study participants received an information brochure illustrating the study results in non-professionals language. These results were also communicated on websites and social media like twitter and Facebook. Furthermore, meetings were organized to inform the society about the obtained research results on improving muscle lipid-turnover and insulin resistance in type 2 diabetes patients by using supplements and pharmacological compounds. Study participants were invited to these meeting as well as other people interested in research and or type 2 diabetes treatment.

ABOUT THE AUTHOR

Yvo Op den Kamp was born on February 7th 1989 in Geleen, the Netherlands. After finishing secondary education at the Trevianum College Sittard in 2007, he enrolled in the bachelor Biomedical Sciences at the Maastricht University. After successfully completing his Bachelor study in 2012, Yvo was admitted to the master program Physician-Clinical Investigator at Maastricht University. During his Master's education, Yvo participated in an extra-curricular internship at the gastrointestinal department in Tribhuvan Teaching Hospital Kathmandu, Nepal. He performed his Master's thesis at the department of Rheumatology at the Maastricht University Medical Center in Maastricht investigating the presence of contributors to secondary and metabolic bone disease (SECOB) in fracture liaison service-patients after a recent clinical fracture. After graduating in August 2016, he started working as medical doctor at foundation Daelzicht and Koraal in Heel.

In February 2017, Yvo started his PhD at the Maastricht University Medical Center in the department of Nutrition and Movement Sciences under the supervision of Prof. Patrick Schrauwen, Dr. Bas Havekes and Dr. Esther Phielix. The research conducted encompassed human studies focused on improving muscle lipid-turnover in insulin resistance and type 2 diabetes via supplementation or pharmacological compounds.

After finalizing his PhD in January 2021, Yvo will continue his career as medical doctor, providing healthcare for intellectually disabled individuals at Op de Bies Landgraaf.

LIST OF PUBLICATIONS

1. **Op den Kamp YJM**, De Ligt M, Dautzenberg B, Kornips E, Esterline R, Hesselink MKC, Hoeks J, Schrauwen-Hinderling VB, Havekes B, Oscarsson J, Phielix E, Schrauwen P. Effects of the SGLT2 inhibitor dapagliflozin on energy metabolism in patients with type 2 diabetes: a randomized, double-blind crossover trial. *Diabetes Care. 2021; doi:10.2337dc20-2887*

2. **Op den Kamp YJM**, De Ligt M, Dautzenberg B, Kornips E, Jorgensen J, Schaart G, Esterline R, Hoeks J, Schrauwen-Hinderling VB, Havekes B, Koves T, Muoio D, Hesselink MKC, Oscarsson J, Phielix E, Schrauwen P. Treatment with SGLT2 inhibitor dapagliflozin alters skeletal muscle acylcarnitine species and increases IMCL in patients with type 2 diabetes. *In preparation.*

3. **Op den Kamp YJM**, Phielix E, Schrauwen P. Supplements and medication for improving lipid-turnover, alleviating IMCL and improving insulin sensitivity.

4. Bruls YMH, **Op den Kamp YJM**, Veeraiah P, Phielix E, Havekes B, Schaart G, Moonen-Kornips E, Wildberger JE, Hesselink MKC, Schrauwen P, Schrauwen-Hinderling VB. Carnitine supplementation improves insulin sensitivity and skeletal muscle acetylcarnitine formation in type 2 diabetes patients. *Submitted*

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