TARGETING HUMAN INSULIN SENSITIVITY

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TARGETING HUMAN INSULIN SENSITIVITY

a cool, an active, and a compound-based approach

Carlijn Remie

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TARGETING HUMAN INSULIN SENSITIVITY

a cool, an active, and a compound-based approach

PROEFSCHRIFT

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Promotoren

Prof. Dr. P. Schrauwen

Prof. Dr. M.K.C. Hesselink

Copromotor

Dr. E. Phielix

Beoordelingscommissie

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

Dr. D.P. Blondin (Université de Sherbrooke, Québec, Canada)

Prof. Dr. M.C.G.J. Brouwers

Prof. Dr. L.J.C. van Loon

Dr. H.P. van der Ploeg (Amsterdam UMC)

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CHAPTER 1

General introduction and outline

THE OBESITY PROBLEM

The worldwide prevalence of overweight and obesity have dramatically increased in the past decades (1). This development is also reflected in the Dutch population. In 1990, 35% of the adult Dutch population was overweight, of which 6% was obese. In 2018, these numbers increased to 50% being overweight, of which 15% was obese (2). Overweight and obesity are recognized as major contributing factors to the development of type 2 diabetes mellitus (T2DM), cardiovascular diseases and non-alcoholic fatty liver disease (3). In 2016, T2DM and cardiovascular diseases were part of the top 5 worldwide causes of death in the age group above 50 years (4). Despite these alarming numbers, the prevalence of overweight and obesity is expected to continue to rise even further in the coming years (5). Given the fact that obesity was once considered a high-income country problem and nowadays these trends appear in low- and middle-income countries as well (1), it has become a worldwide problem. The fundamental cause of obesity is a change in lifestyle leading to a positive energy balance with energy intake exceeding energy expenditure for a prolonged period of time, with body mass gain as a consequence. The underlying explanation for this positive energy balance is in the majority of cases a combination of a high-caloric diet and low levels of physical activity.

INSULIN RESISTANCE AND TYPE 2 DIABETES

In 2018, it was estimated that around 500 million adults worldwide and almost 1.2 million adults in the Netherlands were diagnosed with diabetes (6, 7). Of these numbers, around 90% can be accounted to type 2 diabetes mellitus (T2DM). T2DM is part of the top 3 diseases with the highest disease burden in the Netherlands (8). The prevalence of T2DM in the Dutch population has been estimated to increase by 34% in the period 2015-2040 (9). Likewise, the prevalence worldwide will increase as well, with the greatest expected increase in low-income countries (6), rendering T2DM a serious worldwide health problem.

T2DM is characterized by insulin resistance in peripheral tissues, most notably skeletal muscle, liver, and adipose tissue (see **Figure 1**). As a result, pancreatic insulin secretion will rise in an attempt to compensate for the insulin resistance. During the initial stage of this disease, hyperinsulinemia serves to maintain normoglycemia. Eventually, in a later disease stage, insulin production in the pancreas declines and plasma glucose levels begin to rise. Long term insulin resistance causes chronically increased plasma glucose levels (hyperglycemia), originating from compromised insulin mediated suppression of hepatic glucose output as well as from blunted insulin-stimulated glucose uptake in skeletal muscle (10). Skeletal muscle accounts

for the major part (~80%) of postprandial insulin-mediated glucose disposal (11). Glucose uptake in skeletal muscle occurs via glucose transporter type 4 (GLUT4) translocation from intracellular stores to the cell membrane. Once glucose has entered the cell it can be directed towards oxidation in the mitochondria or be stored as glycogen. Insulin activates the enzyme glycogen synthase thereby promoting postprandial glycogen storage (10). However, during exercise, GLUT4 translocation occurs independently from insulin (12). The insulin mediated GLUT4 translocation and subsequent glycogen synthesis is compromised in patients with type 2 diabetes (10), contributing to hyperglycemia. Patients with T2DM, however, are not resistant to the stimulatory effects of exercise on GLUT4 translocation (13). Normally, in the liver, insulin suppresses hepatic glucose output. Hepatic glucose production originates from glycogenolysis as well as from gluconeogenesis. In case of insulin resistance, the hepatic glucose production is less suppressed in the postprandial state contributing to hyperglycemia (10). Furthermore, insulin resistance in white adipose tissue leads to a decrease in insulin mediated lipid storage and a decreased suppression of lipolysis, with a consequent rise of plasma fatty acid levels (10). This spillover of fatty acids from the white adipose tissue leads to lipid storage in non-adipose tissues like skeletal muscle and liver, also referred to as ectopic lipid storage (14). This ectopic lipid storage deleteriously affects cell function and again has been associated with insulin resistance and type 2 diabetes (14). Thus, insulin resistance in overweight and obese people forms a major risk for the development of T2DM. Eventually, long term hyperglycemia in T2DM patients, can lead to complications, including cardiovascular diseases, nephropathy, neuropathy and retinopathy (15).

THE ROLE OF MITOCHONDRIA

Mitochondria are cellular organelles which are essential for aerobic energy production. Inside the mitochondria, substrates are converted into ATP with the consumption of oxygen via oxidative phosphorylation. ATP provides chemical energy to fuel vital cellular processes, for example muscle contractions. To adapt to oscillations in energy demand and substrate availability, healthy mitochondria can switch readily between oxidative metabolism of fatty acids and glucose, a process often referred to as metabolic flexibility. In the metabolically inflexible state, the switch from predominantly fatty acids in the fasted state to more glucose oxidation in the postprandial state is blunted. This results in delayed postprandial glucose clearance and to disturbances in glucose homeostasis (16), such as hyperglycemia.

Figure 1. Mechanisms of healthy insulin action and insulin resistance

Furthermore, a compromised mitochondrial function can result in impaired fat oxidation and thereby may play a role in ectopic lipid accumulation (17). It has been reported that mitochondrial number and function decline with normal aging (18, 19) and a compromised mitochondrial function has been associated with insulin resistance, type 2 diabetes and cardiovascular diseases (20, 21).

WELL KNOWN INTERVENTION STRATEGIES TO IMPROVE INSULIN SENSITIVITY

In many cases, obesity can be prevented or treated through lifestyle interventions. Exercise and caloric restriction are the two most commonly studied and powerful lifestyle interventions for counteracting obesity and metabolic disturbances including T2DM (22, 23). Both strategies are suggested to act partly via activation of AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1). Both AMPK and SIRT1 are activated during cellular energy crisis, by changes in energy charge. Downstream activation of AMPK and SIRT1 results in many metabolic adaptations, including enhanced mitochondrial biogenesis and function, mostly through activation of peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) (24-26).

Caloric restriction

Creating a negative energy balance by reducing energy intake (caloric restriction) results in weight loss and is the most effective intervention strategy to increase lifespan in a wide range of species, including humans (27). Regarding the metabolic benefits, several intervention studies in overweight humans investigated six months of caloric restriction by 25% reduction in energy intake and reported reduced fasting insulin levels (28), increased gene expression of SIRT1 and PGC-1 α (28, 29), decreased hepatic lipid accumulation (30), increased insulin sensitivity (30) and enhanced mitochondrial biogenesis (29). These studies were accompanied by 10% weight loss. Weight loss has been extensively recognized to improve insulin sensitivity and mitochondrial function by creating a negative energy balance and reducing ectopic lipid storage (31, 32).

Exercise

Exercise training is one of the most potent ways to promote peripheral glucose disposal and to increase muscle mitochondrial function. Numerous human intervention studies have examined the effect of a wide range of exercise training programs on markers of insulin sensitivity and mitochondrial function in humans with obesity (33-36), patients with T2DM (33, 34) and aged people (37), some studies combined with weight loss (32). Exercise exerts significant improvements in glucose metabolism and insulin sensitivity. During an exercise session, the increased skeletal muscle energy demand and concomitant AMPK activation promotes GLUT4 translocation and stimulates substrate oxidation (38). In addition, several post-exercise adaptations occur and share the common purpose to replenish fuel stores, particularly muscle and liver glycogen. To illustrate, increased membrane bound GLUT4 in skeletal muscle and improved insulin sensitivity of skeletal muscle and liver have been observed in lean healthy individuals up to 48 hours after the last exercise session (38, 39).

INVESTIGATION OF NOVEL STRATEGIES TO IMPROVE INSULIN SENSITIVITY

Although these strategies seem very effective, long term compliance to dietary changes or adherence to exercise programs are usually very low in the general population (40, 41). Therefore, novel strategies to improve human metabolic health, affecting insulin sensitivity and mitochondrial function, are urgently needed. This has led to a search for novel intervention strategies, which can initiate the beneficial metabolic health effects similar to those from caloric restriction and exercise training. Within this thesis three novel approaches will be investigated (see **Figure 2**). These approaches will be briefly introduced below.

Promoting NAD⁺ metabolism

Nicotinamide adenosine dinucleotide (NAD) plays a central role in energy metabolism. NAD occurs in the oxidized form as NAD⁺ or in the reduced form as NADH. NAD serves as an electron carrier in the oxidation of substrates. High ratios of NAD⁺/NADH reflect a low cellular energy charge and induces activation of sirtuins. Sirtuins are NAD⁺-dependent deacetylating enzymes that function as metabolic regulators (42). Seven members of the sirtuin family (SIRT1-7) have been identified in humans, of which SIRT1 is the most widely studied family member. SIRT1 is activated in situations of energy stress, such as caloric restriction and exercise (43) and NAD⁺ is the rate limiting substrate for SIRT1 (44). Thus, SIRT1 is a potentially powerful molecular target for the prevention and treatment of obesity and T2DM.

Recently, NAD⁺ increasing compounds have been examined with respect to their SIRT1 activating potential. The increase in NAD⁺ can be achieved by inhibition of NAD⁺ degradation pathways as well as by activating NAD⁺ synthesis pathways. NAD⁺ can be synthesized de novo from the essential amino-acid tryptophan, which is present in our diet. However, the main source of NAD⁺ derives from salvage pathways, which requires uptake of NAD⁺ precursors from our diet. Several NAD⁺ precursors have been identified, including nicotinic acid (NA), Acipimox, nicotinamide (NAM), nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR). The use of NA and the NA-analog Acipimox dates back to the 1980's and was used to treat hyperlipidemia (45). However, more recent studies have shown that Acipimox is also capable of increasing cellular NAD⁺ levels and enhanced mitochondrial function in humans are reported (46), suggesting that supplementation with NAD⁺ precursors is a promising strategy to improve human metabolic health. Most of these nicotinamide related compounds, however, have been linked to unwanted side effects (flushing) and are therefore not preferable targets for the treatment of chronic metabolic diseases (47, 48). NMN and NR can be considered a new generation of NAD⁺ precursors that do not provoke these side effects. In animal models, NMN and NR supplementation have been reported to improve oxidative metabolism, mitochondrial function and insulin sensitivity (49-53).

Since 2016, NR supplements have been available for human use and have been shown to increase circulatory NAD⁺ levels (54). In 2017, the pharmacokinetics and human safety of NR supplementation were described (55, 56). This opens new avenues for human clinical trials to examine if NR supplementation can improve insulin sensitivity, mitochondrial function and other metabolic health related parameters.

Cold acclimation and brown adipose tissue

Besides targeting NAD⁺ metabolism, another approach to improve human metabolic health is cold exposure. Physiological responses to cold exposure serve to maintain core body temperature stable at ~37°C include: 1) insulative responses by peripheral vasoconstriction and 2) metabolic responses by increasing energy expenditure. A fundamental component of human physiology is herein important: namely that coupled (ATP production) and uncoupled mitochondrial respiration coincides with heat release. Several components of energy expenditure may contribute to heat production, including shivering thermogenesis, non-shivering thermogenesis, diet induced thermogenesis and activity-induced thermogenesis.

Heat production through muscle shivering thermogenesis is a well-known response to acute cold exposure in humans and includes involuntary muscle contractions. Brown adipose tissue (BAT) is responsible for the major part of non-shivering thermogenesis during cold exposure in cold-acclimated small mammals (57). BAT is a tissue enriched with a specific uncoupling protein 1 (UCP-1). In response to cold stimulus, this protein is able to uncouple the proton gradients across the inner mitochondrial membrane and thereby generating heat rather than ATP. Since the thermogenic contribution of BAT is much larger in newborns and small mammals, compared to adult humans, it was for a long time believed that non-shivering thermogenesis through BAT only had a minor contribution to thermogenesis in adult humans (58). However, since the rediscovery of the presence of cold-inducible BAT in adult humans in 2009 (59), activation of BAT has been considered a potential strategy to increase human energy expenditure.

The volume and activity of human BAT are inversely related to body fat content and decrease with age (60). It is therefore expected that the reactivation and recruitment of BAT may protect against the onset of obesity and related metabolic disorders such as T2DM. Cold exposure is the most powerful and physiological stimulus for BAT activation, both in small rodents and in humans (61). In fact, research revealed that acute cold exposure in humans increases whole body resting energy expenditure and improves insulin sensitivity, accompanied by elevated BAT activity (62). Acute cold exposure (hours) increases BAT activity (59, 63-66), while longer-term exposure (days/weeks) can also trigger BAT volume expansion (67, 68). In case people are exposed to cold under laboratory conditions for a sustained period, this is referred to as cold acclimation. Cold acclimation studies have reported increased BAT volume as well as elevated BAT activity (69). Furthermore, seasonal variations in basal metabolic rate have been reported with higher values during winter- compared to summertime (70). However, not all beneficial effects of cold exposure on human

metabolism can fully be explained by BAT activation and hence brings up the question if BAT is the only site of non-shivering thermogenesis. Some studies have proposed that, in addition to shivering, skeletal muscle could also play a role in non-shivering thermogenesis (71-74). A cold acclimation study in patients with T2DM has reported improved skeletal muscle glucose uptake and insulin sensitivity (72). However, the molecular underlying causes are not fully understood yet.

Reducing sedentary behaviour

Another effective strategy to increase energy expenditure is exercise, which improves insulin sensitivity and mitochondrial function, as previously described. Exercise, however, should not be confused with physical activity. Physical activity is defined as *“any bodily movement produced by skeletal muscles that requires energy expenditure”* (75). Exercise is a subcategory of physical activity and is defined as *“a subset of physical activities that is planned, structured, repetitive and has as a final or an intermediate objective to improve or maintain physical fitness”* (75). Physical activity can be subdivided based on intensities expressed in metabolic equivalents (METs). MET is the ratio of energy expenditure during a certain activity to the energy expenditure in rest. One MET is defined as 1 kcal/kg/hour and is roughly equivalent to the energy cost of sitting quietly (76). Low-intensity physical activity is defined as activities between 1.5 and 2.9 METs and includes daily activities such as standing, light-intensity walking and light-intensity household activities. Moderate-to-vigorous physical activity is defined as activities ≥ 3.0 METs and includes activities such as brisk walking, cycling, heavy household work, and exercise. The WHO advises to perform 150 min/week of moderate-to-vigorous physical activity (77). Worldwide, 31% of the adult population did not meet this recommendation in 2008, with women being more physically inactive (34%) than men (28%) (78). Data obtained in 2016 revealed that 81% of children aged between 11 and 17 years worldwide were insufficiently physically active in 2016 (79). This indicates that the problem of low physical activity levels will remain a major health problem in the future.

This problem of low physical levels is partly due to an increase in sedentary behaviour. Sedentary behaviour is defined as *“any waking behaviour characterized by an energy expenditure ≤ 1.5 METs, while in a sitting, reclining or lying posture”* (80). Examples of sedentary behaviour include tv viewing, gaming, reading, desk work and driving a car. Sedentary behaviour has frequently been associated with obesity (81) and many chronic diseases including T2DM (82, 83), cardiovascular diseases (82, 84), and cancer (82). Furthermore, sedentary behaviour has been associated with higher mortality rates (85). Adults in the Netherlands spend on average 35% of their time

sleeping, 40% sedentary and only 25% physically active (86). Compared to healthy individuals, the amount of time spend sedentary is higher in the obese population and even higher in patients with T2DM (86).

One of the questions that remains to be answered is whether sitting time should be replaced by low-intensity physical activities such as standing and walking, or by moderate-to-vigorous physical activities such as exercise. It has already been shown that replacement of sitting time by standing and walking, as well as by exercise, improves glucose and lipid metabolism, including markers of insulin sensitivity (87-89). Whether these two sitting replacement strategies act via the same underlying pathway is still unknown.

Figure 2. Proposed working mechanism of novel strategies to improve human metabolic health

OUTLINE OF THIS THESIS

The aim of this thesis is to investigate novel strategies to improve human metabolic health, affecting insulin sensitivity and mitochondrial function. Three human intervention studies have been performed which line up the different chapters of this thesis (see **Figure 2**). In chapter 2, the effect of nicotinamide riboside supplementation – a NAD⁺ precursor – on human metabolic health is described, with a focus on insulin sensitivity and muscle mitochondrial function. Chapter 3 describes the effect of nicotinamide riboside on brown adipose tissue activity. Chapter 4 of this thesis addresses the effect of cold acclimation on human insulin sensitivity and postprandial substrate metabolism. In chapter 5, the effect of replacing sedentary behavior by standing and walking compared to a single exercise bout per day are presented with respect to human insulin sensitivity and muscle mitochondrial function. In chapter 6, all findings of the previous parts will be discussed, and overall conclusions of this thesis will be drawn.

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CHAPTER 2

Nicotinamide riboside supplementation alters body composition and skeletal muscle acetylcarnitine concentrations in healthy obese humans

Carlijn M.E. Remie, Kay H.M. Roumans, Michiel P.B. Moonen, Niels J. Connell, Bas Havekes, Julian Mevenkamp, Lucas Lindeboom, Vera H.W. de Wit, Tineke van de Weijer, Suzanne A.B.M. Aarts, Esther Lutgens, Bauke V. Schomakers, Hyung L. Elfrink, Rubén Zapata-Pérez, Riekelt H. Houtkooper, Johan Auwerx, Joris Hoeks, Vera B. Schrauwen-Hinderling, Esther Phielix, Patrick Schrauwen

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ABSTRACT

Background: Nicotinamide riboside (NR) is a NAD⁺ precursor that boosts cellular NAD⁺ concentrations. Preclinical studies have shown profound metabolic health effects after NR supplementation.

Objective: Investigate the effects of six weeks NR supplementation on insulin sensitivity, mitochondrial function and other metabolic health parameters in overweight and obese volunteers.

Design: A randomized, double-blinded, placebo-controlled, cross-over intervention study was conducted in thirteen healthy overweight and obese men and women. Participants received six weeks NR (1000 mg/day) and placebo supplementation, followed by broad metabolic phenotyping, including hyperinsulinemic-euglycemic clamps, magnetic resonance spectroscopy, muscle biopsies, and assessment of *ex vivo* mitochondrial function and *in vivo* energy metabolism.

Results: Markers of increased NAD⁺ synthesis – NAAD and MeNAM – were elevated in skeletal muscle after NR compared to placebo. NR increased body fat free mass (62.65 ± 2.49 vs. 61.32 ± 2.58 , 1.34 ± 0.50 % in NR, placebo, and the change respectively, $p=0.02$) and increased sleeping metabolic rate. Interestingly, acetylcarnitine concentrations in skeletal muscle were increased upon NR (4558 ± 749 vs. 3025 ± 316 and 1533 ± 683 pmol/mg dry weight in NR, placebo, and the change respectively, $p=0.04$) and the capacity to form acetylcarnitine upon exercise was higher in NR compared to placebo (2.99 ± 0.30 vs. 2.40 ± 0.33 and 0.53 ± 0.21 mmol/kg wet weight in NR, placebo, and the change respectively, $p=0.01$). However, no effects of NR were found on insulin sensitivity, mitochondrial function, hepatic and intramyocellular lipid accumulation, cardiac energy status, cardiac ejection fraction, ambulatory blood pressure, plasma markers of inflammation or energy metabolism.

Conclusion: NR supplementation of 1000 mg/day for six weeks in healthy overweight and obese men and women increased skeletal muscle NAD⁺ metabolites, affected skeletal muscle acetylcarnitine metabolism, and induced minor changes in body composition and sleeping metabolic rate. However, no other metabolic health effects were observed.

INTRODUCTION

Nicotinamide riboside (NR) is a naturally occurring vitamin B3 present in the human diet, acts as a NAD⁺ precursor (1), and is suggested to improve mitochondrial function and insulin sensitivity (2). NR acts via activation of the NAD⁺-dependent sirtuin enzyme family, thereby regulating oxidative metabolism (3-7). *In vitro* experiments have shown the successful NAD⁺ restoring capability of NR supplementation and the subsequent increase oxidative gene expression in skeletal muscle cells (8, 9). Results from *in vivo* mouse models have shown improvements in insulin sensitivity and oxidative energy metabolism, including enhanced metabolic flexibility, increased aerobic exercise capacity and indications of improved mitochondrial biogenesis (9-13). In these mouse models NR seems to specifically act on muscle, liver, heart and brown adipose tissue (9, 14). Reports indicate that NAD⁺ metabolism including NAD⁺ concentrations are decreased in the obese and older population (15-17). The number of human interventions with NR is small and the evidence that NR may have beneficial effects in humans is limited. Human pharmacokinetic studies showed increased circulatory NAD⁺ metabolite concentrations in whole blood, PBMC and urine, after various dosages of NR supplementation (18-23), varying from 300 to 2000 mg/day, indicating that NR supplementation is able to increase the NAD⁺ pool in humans. So far, three randomized placebo-controlled NR supplementation studies have been performed in humans, in which the effect of NR supplementation on human metabolic health was investigated. Dollerup et al. (21, 24), investigated the effect of 2000 mg/day NR for 12 weeks in insulin resistant middle-aged obese men. No effects of NR supplementation were found on insulin sensitivity, muscle mitochondrial function or metabolic flexibility (21, 24). In addition, Martens et al. (20), investigated the effect of 1000 mg/day NR for six weeks in healthy normal weight middle-aged and older men and women. Besides a trend in a reduction in a reduced arterial stiffness and lower blood pressure after NR supplementation, no effects were found on a wide variety of outcomes indicative of metabolic function, glucose metabolism, motor function and exercise capacity (20). Furthermore, Elhassan et al. (25) investigated the effects of 1000 mg/day NR for 3 weeks in older men and showed increased skeletal muscle NAD⁺ metabolite concentrations, however again no effect on skeletal muscle mitochondrial function was observed. To date, the effect of NR supplementation on both insulin sensitivity and skeletal muscle mitochondrial function has only been recently published in one human clinical trial (21, 24). Based on the promising preclinical findings reported until 2017, we designed a double blinded randomized placebo controlled cross-over study in which we aimed to investigate the effect of 1000 mg/day NR supplementation on metabolic health in healthy obese and overweight men and women. The primary focus was on insulin sensitivity and muscle

mitochondrial function, and secondary outcomes were related to energy metabolism and skeletal muscle NAD⁺ metabolites. Therefore, our study expands the limited knowledge of the effect of NR supplementation on human metabolic health and aims to investigate the translational value of previous promising preclinical findings.

METHODS

The study was conducted in accordance with the principles of the declaration of Helsinki and approved by the Ethics Committee of the Maastricht University Medical Center. The study was registered at <https://clinicaltrials.gov> (NCT02835664). All participants provided written informed consent before screening.

Participants

Recruitment and data collection took place between December 2016 and December 2018 in Maastricht, The Netherlands. Thirteen participants completed the study, two dropped out because of personal or medical reasons (see **Supplementary Figure 1**). Men and postmenopausal women were included. Participants underwent a screening including assessment of blood biochemistry, electrocardiography, anthropometric measurements and a questionnaire (including Baecke physical activity questionnaire (26)) to evaluate eligibility. Inclusion criteria were 45-65 years of age, BMI 27-35 kg/m², sedentary lifestyle (<3 h exercise per week), non-smoking for at least 6 months, alcohol use of ≤ 2 servings per day, stable body weight for at least 6 months and having no active diseases.

Study design

The study had a randomized controlled, double blinded, placebo controlled, cross-over design. Simple randomization was applied with use of computer-generated random numbers. Each participant received two interventions each of which lasted six weeks. During the first six weeks the participant was randomized to receive either NR supplementation or placebo. This six-week period was followed by a washout period of four to seven weeks. At the end of the washout period the participant crossed over to the treatment they did not receive during the first six weeks. In both intervention arms participants underwent exactly the same tests to determine the effect of the intervention (**Figure 1**). These tests were performed in the end of week five and during week six. During the entire study period no changes in lifestyle (no change in diet, physical activity level or medication or supplement use) were allowed. Compliance was checked on a weekly basis by pill count. Furthermore, a fasted blood

sample was taken, and body weight was monitored. During supplementation periods, any adverse effects and side effects were noted.

Figure 1. Study design

In this cross-over study, participants were randomly assigned to start with six weeks of NR supplementation or six weeks of placebo treatment. After a washout period of four-to-seven weeks, participants entered the other intervention arm such that all participants served as their own control. Participants were studied in week five (day 32) and in week six (day 36 – 40) of each of the two interventions.

Determination of sleeping metabolic rate

At day 36 of each intervention, participants received a standardized dinner at 18:30 h after which they remained fasted and entered a metabolic chamber at 19:30 h. The metabolic chamber is a small room with a bed, toilet, television, computer and access to water in which oxygen consumption and carbohydrate production were measured continuously in sampled room air. During the overnight stay in the respiration chamber sleeping metabolic rate was assessed. Participants decided themselves what time they went to sleep. At 06:00 h the next morning participants were woken up and left the chamber in an overnight fasted state.

Hyperinsulinemic euglycemic clamp

To determine insulin sensitivity, a two-step hyperinsulinemic euglycemic clamp with co-infusion of D-[6,6-²H₂] glucose tracer (0.04 mg/kg/min) was performed on day 37 of both interventions (27). Insulin was infused, starting at 10:30 h, at 10 mU/m²/min for 2.5 h to assess hepatic insulin sensitivity and subsequently increased to 40

mU/m²/min for 2 h to measure whole body insulin sensitivity. Blood was frequently sampled to measure glucose concentrations directly from arterialized blood and tracer-enriched 20% glucose was co-infused at a variable rate to maintain euglycemia (~5.0 mmol/l) and reach steady state condition. During the steady state of 30 minutes, blood samples were collected, and substrate utilization was measured using indirect calorimetry. Due to technical failures, one participant was excluded from the clamp analysis.

Skeletal muscle biopsies

On day 37 of each intervention, a muscle biopsy was taken at 8:30 h after an overnight stay in the respiration chamber from the m. vastus lateralis under local anesthesia (1% lidocaine, without epinephrine) using the Bergström technique (28). The muscle biopsy was divided in several parts. One part was immediately frozen in melting isopentane for biochemical analyses. The remaining part was used for mitochondrial respiration analysis.

Skeletal muscle NAD⁺ metabolites

NAD⁺ content was determined in muscle biopsies using an enzymatic spectrophotometric cycling assay based on the coupled reaction of malate and alcohol dehydrogenases, as previously described (29). NAD⁺ metabolites were measured in muscle biopsies through metabolomics. Freeze-dried muscle tissue (2-4 mg) was transferred to 2 mL tubes, and then 425 µL water, 500 µL methanol and 75 µL of internal standards mixture (see **Supplementary Table 1**) was added to each sample. Samples were homogenized using TissueLyser II (Qiagen; 5 min at 30/s), followed by addition of 1000 µL chloroform and thorough mixing. After centrifugation, the top layer containing the polar phase was transferred to 1.5 mL tubes and dried in a vacuum evaporator at 60 °C. Dried samples were reconstituted in 100 µL methanol/water (6/4, v/v) and analyzed in an Aquity UPLC system (Waters) coupled to an Impact IITM Ultra-High Resolution qTOF mass spectrometer (Bruker). Chromatographic separation of the compounds was achieved using a SeQuant ZIC-chILIC column (PEEK 100 x 2.1 mm, 3 µm particle size, Merck, Darmstadt, Germany) at 30 °C. The LC method consisted in a gradient running at 0.25 mL/min from 100% mobile phase B (9:1 acetonitrile:water with 5 mM ammonium acetate pH 6.8) to 100% mobile phase A (1:9 acetonitrile:water with 5 mM ammonium acetate pH 6.8) in 28 minutes, followed by a re-equilibration step at 100% B of 5 minutes. MS data were acquired both in negative and positive ionization modes in full scan mode over the range of m/z 50-1200. Due to limited sample availability, NAD⁺ content was measured in muscle biopsies of 8 participants and NAD⁺ metabolomics was measured in muscle biopsies of 12 participants.

Skeletal muscle mitochondrial respiration and protein content

From the muscle biopsies, permeabilized muscle fibers were prepared as described elsewhere (30). Thereafter *ex vivo* mitochondrial respiration was determined by measuring oxygen consumption rate upon addition of several substrates using high-resolution respirometry (Oxygraph, OROBOROS Instruments, Innsbruck, Austria) as described previously (31). All measurements were performed in quadruplicate and the integrity of the outer mitochondrial membrane was assessed in every experiment by the addition of cytochrome C (10 $\mu\text{mol/L}$) upon maximal coupled respiration. The average cytochrome C response in the included traces was $1.7 \pm 0.03\%$ and traces with a cytochrome C response above 15% were excluded from statistical analyses. Data is presented per mg wet weight. Oxphos complex protein content was measured in skeletal muscle biopsies of 12 participants as previously described (32).

Skeletal muscle acylcarnitine concentrations

2-4 mg of freeze-dried muscle tissue was homogenized 1 mL 80% acetonitrile containing 50 μL of the internal standards. After centrifugation at 16000g, the resulting supernatant was dried under a stream of nitrogen at 40 °C and derivatized by addition of 1-propanol/acetylchloride (4/1, v/v) during 15 minutes at 60 °C. After evaporation under nitrogen at 40 °C, samples were redissolved in pure acetonitrile. Determination of the propylated acylcarnitines in the medium was performed by mass spectrometry in an Acquity UPLC System (Waters) coupled to a Quattro Premier XE Tandem Quadrupole Mass Spectrometer (Waters).

VO₂peak

Maximal oxygen consumption (VO₂peak) was assessed during an incremental cycling test on an ergometer (Lode Excalibur Sport, Groningen, The Netherlands) (33), in the first week of the first supplementation period. After a warming-up of five minutes at 75 Watt, the power was increased every 2.5 minutes by 50 Watt until 80% of the maximally calculated heart rate was reached, then the power was increased every 2.5 minutes by 25 Watt until exhaustion. The highest average oxygen consumption over 25 seconds was used as the VO₂peak, reflecting physical fitness of the participant.

MR spectroscopy

Proton magnetic resonance spectroscopy (¹H-MRS) was used to quantify intrahepatic lipid content (IHL), intramuscular lipid content (IMCL) and skeletal muscle acetylcarnitine concentrations. Phosphorus magnetic resonance spectroscopy (³¹P-MRS) was used for assessment of phosphocreatine (PCr) to adenosine triphosphate (ATP) ratio as a marker of the energy status and *in vivo* mitochondrial function of the

heart muscle. Magnetic resonance imaging was used for determination of cardiac left ventricle ejection fraction (EF). All measurements were performed on a 3.0T whole body scanner (Achieva Tx, Philips Healthcare, Best, The Netherlands).

IHL & IMCL: IHL quantification took place on day 36 of each intervention at 17:00 h. Participants were fasted for at least 3 hours. Spectra were acquired as described before (34). Values are given as T2 corrected ratios of the CH₂ peak relative to the unsuppressed water peak, expressed as percentage. Consecutive, IMCL was measured in the m. tibialis anterior of the left leg, as reported earlier (34). Values are given as T1- and T2-corrected ratios of the CH₂ peak relative to the unsuppressed water peak, expressed as percentage. Due to analytical problems only 9 participants could be included in the analyses of IMCL.

Acetylcarnitine: Acetylcarnitine concentrations in skeletal muscle were acquired on day 32 of each intervention at 17:00 h in the evening. Participants were fasted for at least 3 hours and were asked to refrain from strenuous physical activity 48 hours prior to the measurement. Resting skeletal muscle Acetylcarnitine concentrations were measured using a T1-editing method, as described earlier (35). Additionally, Acetylcarnitine concentrations were measured after 30 minutes exercise (70% maximal output on an ergometer). Acetylcarnitine values were converted to absolute concentrations as described earlier (36). The creatine peak was used as a reference.

Cardiac PCr/ATP ratio & ejection fraction: PCr/ATP ratio was quantified by ³¹P-MRS on day 37 of each intervention at 06:30 h, using an ISIS sequence. Participants were positioned prone and headfirst in the MRI. A ¹H³¹P surface heart coil was placed beneath the participants chest. The voxel of interest was carefully placed around the left ventricle of the heart. Spectra were acquired during the end-systolic phase (NSA = 96, number of points = 2048, bandwidth = 3000 Hz) with a repetition time of 5-8 heartbeats, depending on heart rate. PCr and ATP resonances were quantified using a custom written MATLAB (MATLAB 2014b, The MathWorks, Inc., Natick, Massachusetts, United States) script and values were corrected for T1 saturation and expressed as ratio of PCr over gamma-ATP. Due to technical errors, only 11 participants are included in the analyses of PCr/ATP ratio. Left ventricular size was measured for 12 participants based of MRI images of the heart. Left ventricular EF was calculated from the end diastolic volume (EDV) and end systolic volume (ESV) according to the Biplane Ellipsoid Model, as described earlier (37).

Ambulatory blood pressure

Ambulatory blood pressure (BP) was measured (Mobil-O-Graph, I.E.M., Stolberg, Germany) on day 38 and 39 of each intervention for two days and one night (36-hours). Mean systolic (SBP) and diastolic (DBP) blood pressure during day time and night time, and night time dipping were calculated as previously described (38). Night was defined as the mean time going to bed of all participants (23:38 h) until the mean time waking up of all participants (8:05 h). Due to technical failures night time BP could only be obtained from 12 participants.

Body composition

Body composition was determined on day 40 of each intervention at 08:30 h after an overnight fast of at least 10 hours. Body mass and body volume were assessed using air-displacement plethysmography using the BodPod device (Cosmed, Italy, Rome) according to the manufacturer's instructions (39) and previously reported (40).

Blood sampling and analyses

Glucose (Hk-CP, Axonlab, Amsterdam, The Netherlands) and free fatty acids (FFA) (NEFA-HR, WAKO chemicals, Neuss, Germany) were analyzed enzymatically in EDTA plasma using a Pentra 400 (Horiba, Montpellier, France). Triglycerides (Sigma, Zwijndrecht, The Netherlands), cholesterol (CHOD-PAP, Roche Diagnostics, Mannheim, Germany) and HDL-C (CHOD-PAP, Roche Diagnostics, Mannheim, Germany) after precipitation of apoB-containing lipoproteins with phosphotungstic acid and magnesium ions, were analyzed in serum also using a Pentra 400. All samples from one participant were analyzed within one run. LDL-C was calculated for 12 participants according to the Friedewald equation (41). In a subset of 7 participants (age 60 ± 3 years; BMI 30.0 ± 1.7 kg/m²; n=2 women) inflammatory cytokine concentrations were measured on a Luminex® 200TM system using an inflammation 20-plex human Procartaplex panel (eBioscience, EPX200-12185-901) containing markers for sE-Selectin; ICAM-1/CD54; IL-1 α ; IL-4; IL-12p70; IL-17A/CTLA-8; IP-10/CXCL10; MCP-1/CCL2; MIP-1 α /CCL3; MIP-1 β /CCL4; sP-Selectin; TNF α .

Calculations

Energy expenditure was calculated based on the measured averaged oxygen and carbon dioxide concentrations in the inspired and expired gasses with the assumption that protein oxidation was negligible, using the Weir equation (42, 43). Sleeping metabolic rate was calculated as the lowest average 3h energy expenditure during the sleeping period. Glucose oxidation and fat oxidation rates were calculated

according to Perronet et al. (42, 43). Steele's single pool non-steady state equations were used to calculate the rate of glucose appearance (Ra) and the rate of glucose disappearance (Rd) during the clamp (44). Volume of distribution was assumed to be 0.160 l/kg for glucose. The change in insulin-stimulated glucose disposal (Δ Rd) was calculated by the difference between Rd under insulin-stimulated conditions and Rd under basal non-insulin-stimulated conditions. Endogenous glucose production (EGP) was calculated as Ra minus exogenous glucose infusion rate. Non-oxidative glucose disposal (NOGD) was calculated as Rd minus carbohydrate oxidation.

Sample size

The sample size was determined based on demonstrating the statistical superiority of NR on insulin stimulated skeletal muscle glucose disposal compared with placebo. Twelve participants were required to achieve 80 % power with an alpha of 5 %, an assumed treatment difference of within person changes of 3.25 μ mol/kg/min, and an assumed SD of within person changes of 3.60 μ mol/kg/min for a one-group paired t-test for a hyperinsulinemic euglycemic clamp. A dropout of 20% was taken into account, so 15 participants were recruited. The expected effect size and SD was based on previous research within our research group (45).

Statistical analyses

Data are reported as mean $\pm$ SE, unless otherwise stated. Data are presented for n=13, unless otherwise indicated. Differences between interventions were analyzed with a two-tailed paired Students t-test for parametric data and with a Wilcoxon test for non-parametric data. A two-tailed $p < 0.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS version 23.0 for MacOS.

RESULTS

Participant population and study compliance

Thirteen healthy overweight and obese men and women (age 59 ± 5 years; BMI 30.2 ± 2.6 kg/m² [mean $\pm$ SD]; 7 women) participated in the study. Participants were non-smokers, had no active diseases, used no medication or supplements interfering with the study outcomes, had a sedentary lifestyle according to the Baecke questionnaire (7.51 ± 1.16 arbitrary units [mean $\pm$ SD]) and an average $\text{VO}_{2\text{peak}}$ of 27.0 ± 5.7 ml/min/kg [mean $\pm$ SD] (see **Supplementary Table 2**). NR at 1000 mg/day was well tolerated and no adverse events or side effects were reported. Surplus NR and placebo supplements were returned by the participants and compliance rate was

calculated as the proportion of capsules ingested relative to the prescribed number. The mean compliance rate during the six-week NR period was $99.3 \pm 1.8\%$ [mean $\pm$ SD] and during the six-week placebo period was $99.1 \pm 2.2\%$ [mean $\pm$ SD]. Participants were instructed to maintain their habitual diet and physical activity pattern during the entire study, this was confirmed by a stable body weight of 85.2 ± 3.8 kg [mean $\pm$ SD] after six weeks of NR supplementation compared to 85.5 ± 3.8 kg [mean $\pm$ SD] upon six weeks placebo ($p=0.55$).

Figure 2. NAD⁺ metabolites in skeletal muscle after NR and placebo supplementation
A: NAD⁺ concentrations measured in skeletal muscle biopsies by enzymatic assay, $n=8$. **B:** NAD⁺ metabolites measured in skeletal muscle biopsies by mass spectrometry, $n=12$. Black bars is NR, open bars is placebo. Data are expressed as mean $\pm$ SE. P values are derived from the analysis of the mean within-person changes and the SEM of the within-group changes. ** $p<0.01$.

NAD⁺ metabolites in skeletal muscle

Compliance was further checked by analysis of NAD⁺-derived metabolites in muscle biopsy samples collected after six weeks of NR and placebo, ~14-16 hours after supplement intake. First skeletal muscle NAD⁺ content was measured. Quantitative analyses of NAD⁺ concentrations using enzymatic cycling assay in skeletal muscle showed that NAD⁺ content was not different between NR and placebo (1.019 ± 0.126 vs. 1.125 ± 0.106 , 0.106 ± 0.105 nmol/mg dry weight in NR, placebo, and the change respectively, $p=0.34$, $n=8$, **Figure 2A**). The lack of increase in NAD⁺ may indicate that NAD⁺ flux is increased without elevated steady state NAD⁺ concentrations. Therefore, we next performed metabolomics to check if NAD⁺ and NAD⁺ metabolites in skeletal muscle were affected by NR. We confirmed that NR supplementation had no effect on skeletal muscle NAD⁺ content itself (0.96 ± 0.15 fold change in NR compared to placebo, $p=0.91$, $n=12$, **Figure 2B**). However, oral NR supplementation significantly increased two main markers of enhanced NAD⁺ metabolism – nicotinic acid adenine dinucleotide (NAAD, 6.8 ± 1.5 fold change $p<0.01$, $n=12$, **Figure 2B**) and

methylnicotinamide (MeNAM, 3.0 ± 0.6 fold change $p < 0.01$, $n=12$, **Figure 2B**), confirming that indeed NR was amplifying skeletal muscle NAD^+ metabolism without affecting steady state. Moreover, NADH, NADP, NADPH, NAM and NMN concentrations remained unchanged ($p=0.73$, $p=0.79$, $p=0.75$, $p=0.25$ and $p=0.97$ respectively, $n=12$, see **Figure 2B**).

Mitochondrial respiration in skeletal muscle

Supplementation with NR did not result in any change in mitochondrial respiration compared to the placebo state. Respiration in the presence of substrate alone (state 2) (malate (M), malate + octanoyl carnitine (MO2) or malate + glutamate (MG2)) was not different between conditions ($p=0.34$, $p=0.19$, $p=0.74$ respectively, **Figure 3A**). Furthermore, ADP stimulated (state 3) respiration on lipid-derived substrate (malate + octanoyl carnitine + ADP (MO3)) and upon complex I substrates (malate + glutamate + ADP (MG3)) was unchanged ($p=0.67$ and $p=0.64$ respectively, **Figure 3B** and **3C**). Respiration upon parallel electron input to both complex I and II (malate + octanoyl carnitine + glutamate (MOG3)) was not different between conditions (49.87 ± 2.80 vs. 50.92 ± 2.44 pmol/mg/s, -1.04 ± 2.58 in NR, placebo, and the change respectively, $p=0.69$, **Figure 3D**). Similar results were observed when succinate was sequentially added in both experiments (MGS3: 74.27 ± 3.27 vs. 72.90 ± 4.64 , 1.37 ± 5.01 pmol/mg/s in NR, placebo, and the change respectively, $p=0.79$; MOGS3: 75.30 ± 3.92 vs. 76.09 ± 3.31 , -0.79 ± 4.36 pmol/mg/s in NR, placebo, and the change respectively, $p=0.86$, **Figure 3D**). Maximal FCCP-induced uncoupled respiration (state u), reflecting the maximal capacity of the electron transport chain, was also unchanged ($p=0.81$, **Figure 3E**). Finally, state 4o respiration (reflecting proton leak) was similar after NR and placebo conditions ($p=0.89$, **Figure 3F**). For a description of the different states, please see: https://www.bioblast.at/index.php/MitoPedia:_Respiratory_states. Mitochondrial OXPHOS protein concentrations (Complex I 0.96 ± 0.21 vs. 0.91 ± 0.15 , 0.05 ± 0.235 A.U. in NR, placebo, and the change respectively, $p=0.84$; Complex II 1.01 ± 0.17 vs. 0.78 ± 0.05 , 0.23 ± 0.20 A.U. in NR, placebo, and the change respectively, $p=0.64$; Complex III 0.94 ± 0.19 vs. 0.92 ± 0.17 , 0.02 ± 0.22 A.U. in NR, placebo, and the change respectively, $p=0.93$; Complex IV 1.02 ± 0.15 vs. 0.94 ± 0.10 , 0.09 ± 0.14 A.U. in NR, placebo, and the change respectively $p=0.55$; Complex V 1.00 ± 0.17 vs. 0.79 ± 0.16 , 0.21 ± 0.17 A.U. in NR, placebo, and the change respectively $p=0.24$) were similar after NR and placebo supplementation, indicating no effect of NR on mitochondrial content (**Figure 3G**).

Figure 3. Skeletal muscle *ex vivo* mitochondrial respiratory capacity after NR and placebo supplementation

A: State 2 respiration upon malate (M), malate + octanoyl carnitine (MO2) and malate + glutamate (MG2). **B:** ADP stimulated state 3 respiration upon lipid derived substrate, malate + octanoyl carnitine + glutamate (MO3). **C:** ADP stimulated state 3 respiration upon complex I substrates, malate + glutamate (MG3). **D:** ADP stimulated state 3 respiration upon parallel electron input to both complex I and II, malate + octanoyl carnitine + glutamate (MOG3), malate + glutamate + succinate (MGS3), malate + octanoyl carnitine + glutamate + succinate (MOGS3). **E:** State u: maximal FCCP-induced uncoupled respiration. **F:** State 4o: Oligomycin induced respiration not coupled to ATP synthesis. **G:** Protein content of individual complexes of the electron transport chain. Black bars is NR, open bars is placebo. N=12. Data are expressed as mean $\pm$ SE.

Acetylcarnitine concentrations in skeletal muscle

Quantification of acetylcarnitine concentration in skeletal muscle, measured by MRS technique in the early evening at 17:00 h after 3 hours fasting, revealed significantly lower baseline acetylcarnitine concentrations under NR supplementation compared to placebo (1.30 ± 0.16 vs. 1.80 ± 0.18 , -0.53 ± 0.19 mmol/kg wet weight in NR, placebo, and the change respectively, $p=0.02$, **Figure 4A**). Maximally stimulated acetylcarnitine concentrations, measured upon exercise, were not different between conditions (4.29 ± 0.29 vs. 4.20 ± 0.27 , 0.00 ± 0.20 mmol/kg wet weight in NR, placebo, and the change respectively, $p=0.67$, **Figure 4A**). Nonetheless, the capacity to increase acetylcarnitine formation, expressed as the change computed as the post exercise value minus the baseline value, was significantly higher in NR compared to placebo (2.99 ± 0.30 vs. 2.40 ± 0.33 , 0.53 ± 0.21 mmol/kg wet weight in NR, placebo, and the change respectively, $p=0.01$, **Figure 4A**). Based on these results, we decided to perform full acylcarnitine analysis in skeletal muscle biopsies taken at 8:30 h after an overnight fast. Remarkably, acetylcarnitine (C2) concentrations were significantly higher under NR supplementation compared to placebo (C2: 4558 ± 749 vs. 3025 ± 316 , 1533 ± 683 pmol/mg dry weight in NR, placebo, and the change respectively, $p=0.04$; **Figure 4B**). No differences were detected in free carnitine (C0), other short chain (C3 to C5), medium chain (C6 to C12) or long chain acylcarnitines (C13 to C20) between NR and placebo ($p=0.25$, $p=0.27$, $p=0.99$ and $p=0.45$ respectively; **Figure 4C**).

Insulin sensitivity and substrate kinetics

Potential effects of NR on whole body and tissue-specific insulin sensitivity were assessed by a two-step hyperinsulinemic-euglycemic clamp. Whole body insulin stimulated glucose uptake, as expressed by the change in glucose disposal (ΔR_d) from baseline to high insulin dose, was not different between NR and placebo ($p=0.98$, **Table 1**). Hepatic insulin sensitivity, reflected by EGP suppression (EGP%) during the low insulin phase, was not affected by NR compared to placebo ($p=0.30$, **Table 1**). NR had no effect on baseline substrate oxidation (carbohydrate oxidation $p=0.84$, and fat oxidation $p=0.67$). Furthermore, insulin stimulated carbohydrate oxidation or suppression of fatty acid oxidation, reflecting metabolic flexibility, was not different between NR and placebo ($p=0.92$ and $p=0.70$) (see **Table 1**). In addition, non-oxidative glucose disposal (ΔNOGD) during low and high insulin phase of the clamp remained unchanged between conditions ($p=0.88$ and $p=0.99$) (see **Table 1**). Plasma FFA concentrations were suppressed by insulin to a similar extent between NR and placebo ($p=0.97$ and $p=0.99$ during low and high insulin phase respectively, **Table 1**), indicating similar white adipose tissue insulin sensitivity.

Figure 4. Skeletal muscle acylcarnitine concentrations measured in the morning and evening after NR and placebo supplementation

A: Acetylcarnitine concentration measured by MRS in skeletal muscle in the evening during rest, after exercise and the capacity to form acetylcarnitine expressed as the difference between rest and exercise. **B:** Free carnitine (C0) and acetylcarnitine (C2) concentrations measured in muscle biopsies in the morning during rest. **C:** Sum of short chain acylcarnitines (SC), sum of medium chain acylcarnitines (MC) and sum of long chain acylcarnitines (LC) concentrations measured in biopsies during rest in the morning. Black bars is NR, open bars is placebo. N=13. Data are expressed as mean $\pm$ SE. P values are derived from the analysis of the mean within-person changes and the SEM of the within-group changes. * $p < 0.05$.

Plasma biochemistry and inflammatory markers

NR supplementation did not affect fasting plasma glucose, triglycerides, total cholesterol, HDL-C, LDL-C, or inflammatory markers, including chemokine, cytokine or cell-adhesion molecule concentrations (**Table 2**). However, interleukin 1 alpha (IL-1 α) tended to be lower after NR supplementation compared to placebo (1.61 ± 0.28 vs. 2.11 ± 0.35 respectively, $p=0.06$, **Table 2**).

Table 1. Insulin sensitivity and substrate kinetics

Parameter	Placebo	NR	Change	p-value
Ra ($\mu\text{mol/kg/min}$) ¹				
Baseline	9.32 $\pm$ 0.33	9.54 $\pm$ 0.78	0.22 $\pm$ 0.72	0.77
Low insulin	11.66 $\pm$ 0.93	11.74 $\pm$ 0.91	0.08 $\pm$ 0.42	0.85
High insulin	36.70 $\pm$ 3.40	37.60 $\pm$ 2.66	0.91 $\pm$ 2.24	0.69
Rd($\mu\text{mol/kg/min}$) ¹				
Baseline	9.90 $\pm$ 0.53	9.52 $\pm$ 0.84	-0.38 $\pm$ 0.97	0.70
Low insulin	12.06 $\pm$ 1.00	12.36 $\pm$ 0.94	0.30 $\pm$ 0.45	0.51
High insulin	36.76 $\pm$ 3.36	36.32 $\pm$ 2.62	-0.45 $\pm$ 2.13	0.84
Delta baseline-low insulin	2.16 $\pm$ 1.07	2.84 $\pm$ 1.01	0.68 $\pm$ 1.03	0.47
Delta baseline-high insulin	26.86 $\pm$ 3.31	26.79 $\pm$ 2.86	-0.07 $\pm$ 2.17	0.98
EGP ($\mu\text{mol/kg/min}$) ¹				
Baseline	9.32 $\pm$ 0.33	9.54 $\pm$ 0.78	0.22 $\pm$ 0.72	0.77
Low insulin	2.70 $\pm$ 0.40	3.38 $\pm$ 0.56	0.68 $\pm$ 0.48	0.18
% suppression low insulin	70.44 $\pm$ 4.57	61.02 $\pm$ 7.05	-9.42 $\pm$ 7.13	0.30
High insulin	-0.04 $\pm$ 0.26	0.51 $\pm$ 0.56	0.55 $\pm$ 0.51	0.30
% suppression high insulin	99.78 $\pm$ 2.52	96.82 $\pm$ 4.65	-2.95 $\pm$ 4.28	0.50
NOGD ($\mu\text{mol/kg/min}$) ¹				
Baseline	5.27 $\pm$ 1.22	4.88 $\pm$ 1.06	-0.40 $\pm$ 1.18	0.74
Low insulin	3.77 $\pm$ 0.92	3.56 $\pm$ 0.75	-0.19 $\pm$ 0.63	0.76
High insulin	21.28 $\pm$ 2.84	20.85 $\pm$ 2.01	-0.43 $\pm$ 2.03	0.84
Delta baseline-low insulin	-1.51 $\pm$ 1.36	-1.30 $\pm$ 0.85	0.21 $\pm$ 1.34	0.88
Delta baseline-high insulin	16.01 $\pm$ 2.81	15.97 $\pm$ 2.04	-0.03 $\pm$ 1.83	0.99
Carbohydrate oxidation ($\mu\text{mol/kg/min}$)				
Baseline	4.42 $\pm$ 0.81	4.58 $\pm$ 0.55	0.16 $\pm$ 0.61	0.84
Low insulin	7.84 $\pm$ 0.69	8.79 $\pm$ 0.78	0.95 $\pm$ 0.76	0.23
High insulin	14.90 $\pm$ 0.96	14.99 $\pm$ 1.15	0.09 $\pm$ 0.89	0.92
Fat oxidation ($\mu\text{mol/kg/min}$)				
Baseline	3.78 $\pm$ 0.23	3.71 $\pm$ 0.20	-0.07 $\pm$ 0.17	0.67
Low insulin	2.73 $\pm$ 0.19	2.55 $\pm$ 0.17	-0.18 $\pm$ 0.21	0.41
High insulin	1.47 $\pm$ 0.23	1.38 $\pm$ 0.22	-0.09 $\pm$ 0.22	0.70
Plasma FFA ($\mu\text{mol/l}$)				
Baseline	555.34 $\pm$ 30.87	581.16 $\pm$ 32.08	25.83 $\pm$ 35.55	0.48
Low insulin	128.55 $\pm$ 21.74	128.03 $\pm$ 19.26	-0.52 $\pm$ 14.19	0.97
High insulin	49.84 $\pm$ 8.26	56.45 $\pm$ 14.80	6.61 $\pm$ 10.04	0.99
Respiratory exchange ratio				
Baseline	0.77 $\pm$ 0.01	0.77 $\pm$ 0.01	0.00 $\pm$ 0.01	0.83
Low insulin	0.82 $\pm$ 0.01	0.83 $\pm$ 0.01	0.01 $\pm$ 0.01	0.37
High insulin	0.91 $\pm$ 0.01	0.91 $\pm$ 0.01	0.00 $\pm$ 0.01	0.86

Ra, rate of appearance; Rd, rate of disappearance; EGP, endogenous glucose production; NOGD, non-oxidative glucose disposal; FFA, free fatty acids. ¹n=12. Data are expressed as mean $\pm$ SE. P values are derived from the analysis of the mean within-person changes and the SE of the within-group changes.

Table 2. Blood biochemistry

Parameter	Placebo	NR	Change	p-value
Glucose (mmol/l)	5.48 ± 0.14	5.44 ± 0.13	-0.04 ± 0.10	0.70
Triglycerides (mmol/l)	1.57 ± 0.35	1.63 ± 0.38	0.06 ± 0.08	0.24
Total cholesterol (mmol/l)	5.54 ± 0.30	5.55 ± 0.35	0.01 ± 0.11	0.99
HDL-C (mmol/l)	1.32 ± 0.12	1.32 ± 0.09	-0.00 ± 0.04	0.99
LDL-C (mmol/l) ¹	3.42 ± 0.18	3.37 ± 0.17	-0.05 ± 0.07	0.52
sE-selectin (pg/ml) ²	32726 ± 5298	34765 ± 4354	686 ± 2068	0.99
sP-selectin (pg/ml) ²	21428 ± 3662	26645 ± 4065	2624 ± 3739	0.58
ICAM-1 (pg/ml) ²	92493 ± 19326	129236 ± 34547	35350 ± 27518	0.58
TNF-α (pg/ml) ²	29.80 ± 6.38	31.85 ± 6.14	2.70 ± 1.97	0.22
IL-1α (pg/ml) ²	2.11 ± 0.35	1.61 ± 0.28	-0.57 ± 0.24	0.06
IL-4 (pg/ml) ²	8.04 ± 1.03	8.86 ± 1.41	0.71 ± 3.63	0.69
IL-12p70 (pg/ml) ²	76.07 ± 2.73	78.89 ± 3.02	1.94 ± 2.82	0.81
IL-17α (pg/ml) ²	6.36 ± 1.36	7.95 ± 2.09	1.76 ± 1.02	0.16
CXCL10 (pg/ml) ²	3.71 ± 0.55	3.39 ± 0.50	-0.61 ± 0.40	0.22
CCL2 (pg/ml) ²	127.39 ± 24.54	144.34 ± 47.86	21.42 ± 40.12	0.69
CCL3 (pg/ml) ²	15.42 ± 8.59	17.04 ± 10.39	2.43 ± 1.92	0.30
CCL4 (pg/ml) ²	23.89 ± 5.98	28.49 ± 7.51	4.52 ± 4.45	0.38

Blood samples were taken in week six of NR supplementation and placebo after an overnight fast.

¹ n=12, ² n=7. Data are expressed as mean ± SE. P values are derived from the analysis of the mean within-person changes and the SE of the within-group changes.

Body composition

After six weeks of NR and placebo supplementation, several changes in body composition were detected. Percentage fat free mass (FFM) was significantly higher after NR compared to placebo (62.65 ± 2.49 vs. 61.32 ± 2.58, 1.34 ± 0.50 % in NR, placebo, and the change respectively, p=0.02, **Figure 5B**). In line with this, percentage fat mass (FM) was significantly lower after NR compared to placebo (37.35 ± 2.49 vs. 38.68 ± 2.58, -1.34 ± 0.50 % in NR, placebo, and the change respectively, p=0.02, **Figure 5B**). However, total bodyweight remained unchanged (p=0.55, **Figure 5A**).

Sleeping metabolic rate

Sleeping metabolic rate (SMR), measured during an overnight stay in a respiration chamber, was higher upon six weeks of NR compared to placebo (6.68 ± 0.30 vs. 6.49 ± 0.31, 0.19 ± 0.08 MJ/day in NR, placebo, and the change respectively, p=0.05, **Figure 5C**). This could be explained by the increase in FFM and sleeping metabolic rate per FFM was not significantly different (0.127 ± 0.003 vs. 0.125 ± 0.003, 0.00 ± 0.00 MJ/FFM/day in NR, placebo, and the change respectively, p=0.48, **Figure 5E**).

Figure 5. Body composition and sleeping metabolic rate after NR and placebo supplementation
A: Bodyweight, fat mass (FM) and fat free mass (FFM) expressed in kilograms (kg). **B:** FM and FFM expressed as percentage (%) of body weight. **C:** Sleeping metabolic rate expressed (SMR) as MJ per day. **D:** SMR corrected for body weight. **E:** SMR corrected for FFM. Black bars is NR, open bars is placebo. Data are expressed as mean $\pm$ SE. N=13. P values are derived from the analysis of the mean within-person changes and the SEM of the within-group changes. * $p < 0.05$.

Ectopic lipid storage

Intrahepatic lipid content, measured by MRS, was not different between NR and placebo conditions (3.4 ± 1.2 vs. 3.4 ± 1.3 , 0.0 ± 0.0 % in NR, placebo, and the change respectively, $p=0.85$). Furthermore, intramyocellular lipid content, measured by MRS technique in the m. tibialis anterior, was not affected by NR supplementation (0.5 ± 0.1 vs. 0.5 ± 0.1 , 0.0 ± 0.1 % in NR, placebo, and the change respectively, $p=0.50$).

Cardiac function

To investigate if NR supplementation could affect cardiac energetics, we determined cardiac PCr/ATP ratios, which however were not affected by NR ($p=0.90$, **Table 3**). No differences were observed in left ventricular end systolic volume (ESV), end

diastolic volume (EDV), stroke volume (SV) and subsequently ejection fraction (EF) between NR and placebo ($p=0.23$, $p=0.72$, $p=0.69$ and $p=0.24$ respectively, **Table 3**). NR supplementation had no effect on 24-hour systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP), and heart rate (HR) ($p=0.56$, $p=0.39$, $p=0.40$, $p=0.60$ and $p=0.60$ respectively, **Table 3**). Separate analyses of day time and night time measurements did not reveal an effect of NR supplementation (see **Table 3**). In addition, night time dipping of SBP and DBP was not affected by NR compared to placebo ($p=0.53$ and $p=0.26$ respectively, **Table 3**).

Table 3. Cardiometabolic health parameters

Parameter	Placebo	NR	Change	p-value
MRS Cardiac left ventricle²				
Cardiac PCr/ATP ratio	1.29 ± 0.11	1.22 ± 0.09	-0.08 ± 0.14	0.90
MRI Cardiac left ventricle¹				
EF (%)	71.2 ± 2.2	68.2 ± 2.1	-3.0 ± 2.4	0.24
ESV (ml)	35.0 ± 4.2	38.9 ± 3.9	4.0 ± 3.6	0.23
EDV (ml)	119.4 ± 8.2	121.2 ± 8.3	1.8 ± 5.1	0.72
SV (ml)	84.4 ± 5.6	82.3 ± 5.6	-2.1 ± 3.8	0.69
Ambulatory BP 36-hour¹				
SBP (mmHg)	124.6 ± 2.4	126.6 ± 3.2	2.0 ± 2.3	0.56
DBP (mmHg)	77.1 ± 1.8	77.9 ± 2.0	0.8 ± 0.9	0.39
MAP (mmHg)	98.9 ± 1.7	100.2 ± 2.1	1.3 ± 1.5	0.40
PP (mmHg)	47.5 ± 2.6	48.6 ± 3.1	1.2 ± 2.2	0.60
HR (bpm)	76.4 ± 3.3	75.5 ± 3.6	-0.9 ± 1.7	0.60
Night time dipping SBP (%)	10.6 ± 2.4	12.4 ± 0.8	1.8 ± 2.8	0.53
Night time dipping DBP (%)	11.9 ± 2.4	14.8 ± 1.9	3.0 ± 2.5	0.26
Ambulatory BP daytime				
SBP (mmHg)	126.9 ± 2.3	129.0 ± 2.9	2.1 ± 2.3	0.54
DBP (mmHg)	79.5 ± 1.7	80.5 ± 1.9	1.0 ± 1.0	0.32
MAP (mmHg)	101.4 ± 1.5	102.7 ± 1.9	1.3 ± 1.4	0.39
PP (mmHg)	47.6 ± 2.7	48.5 ± 3.0	0.9 ± 2.1	0.68
HR (bpm)	78.3 ± 3.2	77.5 ± 3.5	-0.8 ± 1.7	0.67
Ambulatory BP night-time¹				
SBP (mmHg)	113.5 ± 3.4	113.0 ± 3.1	-0.5 ± 3.7	0.90
DBP (mmHg)	69.4 ± 2.6	68.1 ± 2.3	-1.3 ± 1.5	0.41
MAP (mmHg)	89.6 ± 2.7	88.8 ± 2.4	-0.8 ± 2.5	0.75
PP (mmHg)	43.8 ± 2.5	45.2 ± 2.6	1.4 ± 2.3	0.57
HR (bpm)	65.6 ± 2.5	66.6 ± 2.9	1.0 ± 1.9	0.60

PCr, phosphocreatin; ATP, adenosine triphosphate; EF, ejection fraction; ESV, end systolic volume; EDV, end diastolic volume; SV, stroke volume; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; HR, heart rate. ¹n=12, ²n=11. Data are expressed as mean ± SE. P values are derived from the analysis of the mean within-person changes and the SE of the within-group changes.

DISCUSSION

We hypothesized that NR supplementation in humans would increase NAD⁺ availability and thereby would improve a broad range of metabolic health parameters, mainly via improving mitochondrial function. To investigate this hypothesis, we performed a randomized double-blinded placebo controlled cross-over study with detailed metabolic phenotyping in which we provided healthy overweight and obese men and women 1000 mg/day NR for six weeks. In line with our hypothesis, NR supplementation did significantly increase markers of NAD⁺ metabolism – NAAD and MeNAM – in skeletal muscle. This effect was accompanied by small but statistically significant improvements in body composition, sleeping metabolic rate and skeletal muscle acetylcarnitine concentrations, and a trend towards increased circulatory IL-1 α concentrations. No further effects on skeletal muscle mitochondrial function, hepatic and whole-body insulin sensitivity, substrate oxidation, cardiovascular health markers and ectopic lipid accumulation were observed. However, it should be noted that many outcomes have been tested in our study and no adjustments for multiple comparisons were performed, therefore the possibility of false positive findings cannot be excluded. These results suggest that NR, at the dose of 1000 mg/day for six weeks, did have, albeit relatively small, effects on metabolic parameters in humans, but was not effective in boosting muscle mitochondrial function or insulin sensitivity.

Animal studies showed that NR is able to increase plasma and tissue NAD⁺ concentrations (9-14). Also in humans, NR is able to increase circulatory NAD⁺ metabolites after several dosages ranging from 100 mg/day to 2000 mg/day (18-21). Here, we used a dose of 1000 mg/day for six weeks, and in line with other data presented we did not report side effects (18, 20). We investigated if NR supplementation was able to increase the NAD⁺ metabolome in skeletal muscle tissue. In agreement with findings by Elhassan et al. (20) and Dollerup et al. (24), we show that NR supplementation increased NAD⁺ metabolites NAAD and MeNAM in skeletal muscle, but without an increase in total NAD⁺ content itself. NAAD is a highly sensitive biomarker of NR supplementation and increased NAD⁺ synthesis rate in tissues (14). MeNAM is part of the NAD⁺ degradation pathway and is a marker for increased NAD⁺ flux. These results might suggest that NR increases NAD⁺ turnover rate, without affecting steady state NAD⁺ concentrations in skeletal muscle.

We hypothesized that a NR-stimulated increase in NAD⁺ metabolism would lead to an increase in muscle mitochondrial function and a subsequent increase in human insulin sensitivity. However, in contrast to our hypothesis, skeletal muscle

mitochondrial function was not elevated upon NR supplementation. This is in agreement with the findings of Elhassan et al. (25) and Dollerup et al. (24), who also reported no effect of NR on skeletal muscle mitochondrial function. Consistent with the lack of effect on mitochondrial function, we and others (21) did not observe improvements on insulin sensitivity upon NR supplementation. Although the limited duration of our and other studies may explain the lack of effect of NR on insulin sensitivity, we have previously shown that nutritional supplements like resveratrol can increase skeletal muscle mitochondrial function after four weeks of supplementation (45-47). Trammel et al. (48) and Dollerup et al. (21) suggested that the underlying pathway of metabolic improvements observed in obese mice was a decrease in hepatic lipid accumulation. In addition, Dollerup et al. (21) described a decrease in hepatic lipid content in obese men with elevated baseline hepatic lipid content, although this was not significant. Here, we did not observe an effect of NR on hepatic lipid accumulation, which may be contributed to our study population which had in general a healthy hepatic lipid content (i.e. < 5% liver fat).

NR supplementation has also been suggested to improve cardiovascular health (20, 49). Therefore, we here examined the effect of NR on cardiovascular health via detailed cardiovascular phenotyping. In contrast to Martens et al. (20) but in accordance with Conze et al. (23), we did not observe an effect of NR on blood pressure values. Martens et al. observed a decrease in resting SBP and DBP after NR, whereas we measured 36-hour ambulatory blood pressure, which gives a better estimation of blood pressure values and gives a better indication of the risk of cardiovascular events (50, 51). Consistent with the lack of effect of NR on blood pressure we did not find effects of NR on cardiac energy status or cardiac ejection fraction. Of note, the cardiac status of our participants was considered 'healthy', and in rodents also no change in cardiac function after NR supplementation in control mice with a healthy cardiac function could be observed (49).

We reported an improvement in body composition by an increase in percentage fat free mass mirrored by a decrease in percentage fat mass, while body weight remained unchanged. An effect of NR on body composition in humans has not been reported before (20, 21). Interestingly, in six out of seven women NR did increase fat free mass and reduce fat mass whereas this was only the case in one out of six men, suggesting that there might be a gender difference in the effect of NR supplementation on body composition. Consistent with the effect of NR on body composition, we show that the sleeping metabolic rate was also affected by NR supplementation and a higher metabolic rate could potentially lead to a reduction in fat mass. The increase in sleeping metabolic rate was due to an increase in fat free

mass, suggesting that the primary effect of these findings could be an effect of NR on fat free mass. Interestingly, it has previously been shown that NAD⁺ metabolism and homeostasis is involved in maintaining muscle mass (13). Future studies should be designed to investigate if NR supplementation can indeed increase muscle mass in humans and investigate the underlying mechanisms.

Next to an effect of NR on muscle mass, NR supplementation enhanced the exercise-induced increase in acetylcarnitine. Moreover, acetylcarnitine concentrations measured in skeletal muscle biopsies obtained in the morning, showed significantly increased concentrations after NR supplementation. These data suggest that NR is able to increase skeletal muscle acetylcarnitine metabolism, which has been associated with metabolic flexibility and improved metabolic health (36). Remarkably, though, resting acetylcarnitine concentrations measured using MRS three hours after lunch and before the exercise session were significantly lower after NR compared to placebo. It has previously been shown that meal consumption lowers acetylcarnitines in skeletal muscle (52, 53), but why NR would substantiate such meal-induced lowering in acetylcarnitine metabolism cannot be deducted from this study. Interestingly, a recently published study in obese mice showed an effect of combined supplementation of NR with L-carnitine and reported a reduction in fat mass percentage and hepatic steatosis (54), which matches with the positive outcome parameters of our and other studies (9, 21, 48). The exact link between NR metabolism and acetylcarnitine metabolism is however still unknown. Furthermore, the large gap between the clear metabolic improvements upon NR in mice and the lack of effects in humans might derive from the fact that mice studies applied a longer supplementation duration (8 to 15 weeks) (9, 10, 12, 48) compared to short-term supplementation in human trials (3 to 12 weeks) (21, 24, 25, 55).

In conclusion, we here show that NR supplementation of 1000 mg/day for six weeks in healthy overweight and obese men and women increased the NAD⁺ metabolites NAAD and MeNAM in human skeletal muscle and increased skeletal muscle acetylcarnitine metabolism. In addition, NR induced improvements in body composition and increased sleeping metabolic rate. However, no other metabolic health effects were observed. We conclude that NR, at this dose and short duration, may not be beneficial in improving metabolic health in healthy overweight and obese men and women. However, further research is warranted into the effects of long-term NR supplementation on acetylcarnitine concentrations, sex specific improvements in body composition and metabolic health effects in humans.

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The authors' contributions were as follows: CR BH JA JH VSH EP PS designed the study. CR KR MM NC VdW conducted the experiments. BH provided medical responsibility. SA BS HE RZP performed analysis in plasma samples and muscle biopsies. CR JM VdW TvdW SA RZP VSH analyzed data. CR SA EL RZP RH JH LL VSH EP PS interpreted the data. CR EP PS wrote paper and all authors reviewed and accepted the last version of the manuscript. PS had primary responsibility for final content. The authors would like to thank the participants for their commitment to this study and ChromaDex Inc. for providing NIAGEN and placebo capsules for the study. PS 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. The authors declare no conflict of interest relevant to this article.

SUPPLEMENTARY MATERIAL FOR CHAPTER 2

Supplementary Table 1. Internal standard mixture for NAD⁺ targeted metabolomics in skeletal muscle biopsies

Internal standard	Concentration (μM)
Adenosine- ¹⁵ N ₅ -monophosphate	100
Adenosine- ¹⁵ N ₅ -triphosphate	1000
D ₄ -alanine	100
D ₇ -arginine	100
D ₃ -aspartic acid	100
D ₄ -citric acid	100
¹³ C ₁ -citrulline	100
¹³ C ₆ -fructose-1,6-diphosphate	100
Guanosine- ¹⁵ N ₅ -monophosphate	100
Guanosine- ¹⁵ N ₅ -triphosphate	1000
¹³ C ₆ -glucose	1000
¹³ C ₆ -glucose-6-phosphate	100
D ₃ -glutamic acid	100
D ₅ -glutamine	100
¹³ C ₆ -isoleucine	100
D ₃ -leucine	100
D ₄ -lysine	100
D ₃ -methionine	100
D ₆ -ornithine	100
D ₅ -phenylalanine	100
D ₇ -proline	100
¹³ C ₃ -pyruvate	100
D ₃ -serine	100
D ₅ -tryptophan	100
D ₄ -tyrosine	100
D ₈ -valine	100
¹³ C ₅ -nicotinamide adenine dinucleotide	100

Supplementary Table 2. Participant characteristics

Parameter	Mean $\pm$ SD
Gender F/M	7/6
Age (years)	59 $\pm$ 5
Body weight (kg)	87.2 $\pm$ 13.6
Height (m)	1.70 $\pm$ 0.11
BMI (kg/m ²)	30.2 $\pm$ 2.6
VO ₂ peak (ml/min/kg)	27.0 $\pm$ 5.7
Physical activity level (Baecke score)	7.51 $\pm$ 1.16

BMI, body mass index; VO₂peak, peak oxygen consumption.

Supplementary Figure 1. Flowchart participant inclusion

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CHAPTER 3

Increasing NAD⁺ levels through nicotinamide riboside activates *in vivo* brown adipose tissue in mice but not in humans

Emmani B.M. Nascimento*, Michiel P.B. Moonen*, **Carlijn M.E. Remie**, Karim Gariani, Johanna A. Jörgensen, Gert Schaart, Johan Auwerx, Wouter D. van Marken Lichtenbelt, Patrick Schrauwen

* These authors contributed equally

Submitted

ABSTRACT

Elevating NAD⁺ levels systemically improves metabolic health, which can be accomplished via nicotinamide riboside (NR). Previously, it was demonstrated that NR supplementation in high fat diet (HFD)-fed mice decreased weight gain, normalized glucose metabolism and enhanced cold tolerance. As brown adipose tissue (BAT) is a major source of thermogenesis, we hypothesize that NR stimulates BAT in mice and humans. HFD-fed mice were supplemented with 400 mg/kg/day NR and subsequently exposed to cold. Next, *in vitro* primary adipocytes derived from human BAT biopsies were pretreated with 50 μ M or 500 μ M NR prior to measuring mitochondrial uncoupling. Finally, human volunteers were supplemented with 1000 mg/day NR for six weeks to determine whether BAT activity increased, as measured by [18F]-FDG uptake via PET-CT. NR supplementation in HFD-fed mice decreased BAT mass and adipocyte cell size. Cold exposure further decreased adipocyte cell size on top of that achieved by NR alone. In adipocytes derived from human BAT, NR enhanced *in vitro* norepinephrine (NE)-stimulated mitochondrial uncoupling. However, NR supplementation in human volunteers did not alter BAT activity or cold induced thermogenesis. Our research demonstrates the need for translational research in order to better understand the differences in NAD⁺ metabolism between mouse and human.

INTRODUCTION

Brown adipose tissue (BAT) has been proposed as a promising target to stimulate energy expenditure in humans with obesity or type 2 diabetes. The presence of uncoupling protein 1 (UCP1) in mitochondria of BAT enables heat production instead of ATP synthesis while using glucose and fatty acids as fuel (1). The interest in human BAT was sparked by the discovery of active BAT in adult humans (2-5) and high BAT activity is associated with healthy whole-body metabolism. Cold exposure is the most effective way to stimulate BAT activity in humans (6). Prolonged cold exposure may, however, not be an attractive treatment option for many people and thus pharmacological alternatives are actively explored. In this context, we and others have shown that bile acids stimulate BAT activity in mice (7) and healthy humans (8), however, it is not known whether this approach is effective in human metabolic disease. In addition to its direct effect on BAT, bile acids have recently been shown to induce browning of the subcutaneous white adipose tissue (3) through the bile acid-responsive membrane receptor TGR5 (9). This would enhance the thermogenic activity of WAT and promote the use of glucose and fatty acids for mitochondrial uncoupling, which is relevant in humans with disturbed glucose and/or fatty acid metabolism (e.g. obesity, type 2 diabetes).

Another approach to activate BAT involves activation of the beta-adrenergic receptor via administration of agonists, like mirabegron. However, beta-adrenergic stimulation at doses high enough to activate BAT also affects cardiac function by increasing heart rate and blood pressure, due to beta-adrenergic receptors in the heart (10). Therefore, other more specific and safer pharmacological strategies are warranted that stimulate BAT activity in humans.

The vitamin B3 analogue nicotinamide ribose (NR) is a NAD⁺ precursor that has been shown to increase the activity of sirtuin (SIRT) 1 and 3 (11). *In vitro*, NR boosts NAD⁺ levels (12) also resulting in elevated SIRT1/3 activity *in vivo* (11). In obesity models, NR protected high fat diet (HFD)-fed mice from weight gain and glucose intolerance (11, 13). Furthermore, NR supplementation in various rodent models was able to counteract harmful effects of brain damage (14), Alzheimer's disease (15), senescence (16) and muscular dystrophy through increased mitochondrial function (17). NR supplementation also stimulated mitochondrial biogenesis in muscle and BAT in a mouse model of mitochondrial myopathy (18). Interestingly, in mice NR safeguards body temperature following a cold challenge (11) thus implicating the involvement of BAT. These findings give rise to two additional questions: (1.) Is beta-adrenergic stimulated BAT activity altered in mice *in vivo* and in cultured adipocytes

derived from human BAT following NR?; and (2.) Does NR enhance BAT activity in humans?

Therefore, we here assessed how NR affects BAT in a *in vivo* HFD-fed mouse model. Since murine BAT is different from human BAT and the effect of NAD⁺ boosting on human BAT remain unclear, we also investigated the effects of NR on human BAT by using *in vitro* primary cultured adipocytes derived from human BAT biopsies. Finally, we performed a first human clinical trial in which we supplemented human volunteers with 1000 mg/day NR in order to examine the effects on *in vivo* BAT activity in humans.

METHODS

Animal experiments

Male C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA) and were housed under a 14-hour light, 10-hour dark cycle at 21 °C and had ad libitum access to water and food throughout the experiment. From the age of eight weeks, mice were split into four groups of ten animals. All animals received a high fat diet (D12492) for four weeks from Research Diets Inc. (New Brunswick, NJ). Half of animals were fed with pellets containing vehicle (double-distilled water; ddH₂O) or NR-supplemented by providing NR (400 mg/kg/day) for four weeks as previously described (19). Mouse body weight was assessed weekly. Cold test was performed as described (20) in seven animals on HF diet alone and in seven animals receiving HF and NR. The rest of the animals was maintained at room temperature. In cold-test groups, mice were anesthetized with sodium pentobarbital after six hours of cold exposure (i.p. injection, 50 mg/kg body weight) and euthanized by blood sampling via cardiac puncture). In the RT group, mice were anesthetized at the same time with sodium pentobarbital (i.p. injection, 50 mg/kg body weight) and euthanized by blood sampling via cardiac puncture). BAT was snap frozen in liquid nitrogen stored at -80 °C. For imaging purposes, BAT was frozen in melting isopentane or stored in 4% formaldehyde. All animal experiments were carried according to national Swiss and European Union ethical guidelines and approved by the local animal experimentation committee of the Canton de Vaud under license #2868.

Lipolysis assay

Murine BAT was isolated and tissue explants were minced and incubated with lipolysis medium at 37 °C and 5% CO₂. After 2 hours, media was collected and incubated for of five minutes at 37 °C with free glycerol reagent from Sigma-Aldrich (St. Louis, MO). Following absorption was measured at 540 nm as previously

described (21). In cultured adipocytes derived from human BAT and WAT, glycerol release was determined using the enzyChrom™ adipolysis assay kit from BioAssay systems (Hayward, CA) according to the manufacturer's instructions.

Histology

BAT samples were fixed in buffered 4% formaldehyde overnight and embedded in paraffin. 5 µm thick serial sections were made from paraffin embedded tissue then stained with hematoxylin and eosin (HE). Another set of BAT samples were embedded in Thermo Scientific™ Shandon™ Cryomatrix™ embedding resin and snap-frozen, for two minutes in isopentane cooled in liquid nitrogen, before being placed on dry ice. 10 µm cryosection were cut from biopsies frozen in isopentane. Sections were blocked prior to permeabilization with 0.5% triton X-100. Following sections were incubated with antibodies against UCP1 or TOMM20 from Abcam (Cambridge, United Kingdom). After incubation with the appropriate secondary antibody, sections were mounted using Mowiol prior to imaging.

Culture of human primary adipocytes

The collection of BAT and WAT biopsies in patients was reviewed and approved by the ethics committee of Maastricht University Medical Center (METC 10-3-012, NL31367.068.10, NCT03111719). Isolation of the stromal vascular fraction and differentiation of cultured adipocytes derived from human BAT and WAT have been described previously (8). In short, collected cells from the stromal vascular fraction were grown to confluence. Differentiation was initiated by a cocktail containing biotin (33 µM), pantothenate (17 µM), insulin (100 nM), dexamethasone (100 nM), IBMX (250 µM), rosiglitazone (5 µM), T3 (2 nM) and transferrin (10 µg/ml). Cells were transferred to maintenance medium consisting of biotin (33 µM), pantothenate (17 µM), insulin (100 nM), dexamethasone (10 nM), T3 (2 nM) and transferrin (10 µg/ml) until lipid-accumulating adipocytes had formed.

Mitochondrial respiration

Mitochondrial respiration in cultured adipocytes derived from human BAT and WAT has been described before (8). In short, adipocytes derived from human BAT and WAT were differentiated in XF96 well plates. Oxygen consumption rates were measured using the XF96 extracellular flux analyzer from Seahorse Biosciences (North Billerica, MA). Cells were incubated for 1 hour at 37 °C in unbuffered DMEM (2 mM GlutaMAX, 1 mM sodium pyruvate and 25 mM glucose). Basal oxygen consumption was measured followed by injection of 2 µM oligomycin subsequently followed by injection of the compounds of interest. When indicated cells were

preincubated with NR for 24 hours prior to the start of the experiment. Data is plotted as a percentage compared to uncoupled respiration following oligomycin.

RNA isolation and gene expression analysis

Total RNA was extracted from cultured adipocytes derived from human BAT and WAT using the miRNEasy kit from Qiagen (Hilden, Germany) according to the manufacturer. cDNA was created using the high capacity RNA-to-cDNA-kit from Applied Biosystems (Foster City, CA). Gene expression data was normalized to TATA box-binding protein (TBP) and further analyzed using the $2^{-\Delta\Delta Ct}$ method. Primers for SIRT3 (Hs00953477_m1), SOD2 (Hs00167309_m1) and UCP1 (Hs00222453_m1) were from Applied Biosystems (Foster City, CA). SYBR-green qPCR primers for PGC1a: forward primer 5'-TGCTGAAGAGGGAAAGTGAGCGATTAGTTGA-3', reverse primer 5'-AGGTGAAAGTGTAATACTGTTGGTTGA-3'; SIRT1: forward primer 5'-AGAGCCTCACATGCAAGCTCTAG-3', reverse primer 5'-GCCAATCATAAGATGTTGCTGAAC-3'; TFAM: forward primer 5'-TTCCAAGACTTCATTTCATTGTC-3', reverse primer 5'-GATGATTCGGCTCAGGGAAA-3'. Primers for TBP have been described previously (22).

Western blot analysis

Protein was extracted using lysis buffer (50 mM Tris, 1 mM EDTA, 1% NP40, 5 mM nicotinamide, 1 mM sodium butyrate, 150 mM KCl, protease inhibitors [pH 7.4]). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blocking and antibody incubations were performed in 5% BSA. UCP1 antibody was from Abcam (Cambridge, UK). Antibody detection reactions were developed by enhanced chemiluminescence from Advansta (Menlo Park, CA) using imaged using the c300 imaging system from Azure Biosystems (Dublin, CA). Cultured adipocytes derived from human BAT and WAT were harvested in Laemmli buffer. Proteins were separated using SDS-PAGE followed by transfer to nitrocellulose membranes. Proteins were detected and quantified using the Odyssey from LI-COR Biosciences. Antibodies against UCP1 and OXPHOS were purchased from Abcam (Cambridge, United Kingdom). Beta-actin (A5316) was detected using an antibody from Sigma-Aldrich (St. Louis, MO).

NAD⁺ determination

NAD⁺ concentrations were determined using the enzyChrom™ NAD⁺/NADH assay kit from BioAssay Systems (Hayward, CA) according to the manufacturer's instructions.

ATP determination

ATP concentrations were determined using the CellTiter-Glo® Luminescent cell viability assay from Promega (Madison, WI) according to the manufacturer's instructions.

Clinical trial in humans

The ethics committee of Maastricht University Medical Center approved the study protocol (METC 16-30-19, NL58119.068.16, NCT02835664) and all volunteers provided written informed consent. Eight healthy overweight and obese men and postmenopausal women were recruited. Inclusion criteria were 45-65 years of age, BMI 27-35 kg/m², sedentary lifestyle (<3 h exercise per week), non-smoking for at least 6 months, no alcohol use of >2 servings per day, stable body weight for at least 6 months and no active diseases. A randomized controlled, double blinded, placebo controlled, cross-over study with NR supplementation was performed. Volunteers underwent two times a six-week period with oral supplementation of either NR 1000 mg daily (NIAGEN, Chromadex) or placebo (capsules identical to NR in external appearance and number). Supplements were consumed with breakfast (500 mg) and lunch (500 mg). Measurements were performed on the last day of each supplementation period. Participants were fasted for at least 11 hours when entering the test facilities. For the determination of cold-induced BAT activity, volunteers were wrapped in a water-perfused suit (ThermaWrap Universal 3166; MTRE Advanced Technologies Ltd., Yavne, Israel). First, volunteers remained at thermoneutral conditions (32 °C water) for 30 minutes, during which basal metabolic rate was measured by indirect calorimetry (IDEE, Maastricht Instruments, Maastricht, The Netherlands). Thereafter an individualized cooling procedure was started to determine NST as described before (23). In brief, volunteers were cooled down in order to maximize NST after which energy expenditure was measured for 30 minutes. Following, 75 MBq of [18F]-FDG was injected via the intravenous canula. Cold exposure was continued for 60 minutes, while volunteers were instructed to remain lying still. Next, the volunteers were unwrapped and underwent a static [18F]-FDG-PET/CT scan (Gemini TF PET-CT, Philips, The Netherlands). This consisted of a low-dose CT scan (120 kV, 30 mAs) followed by a PET scan. Six to seven bed positions (5 minutes per bed position) were used, to cover the area from the skull to the iliac crest. Tracer uptake was determined with the PET scan while the CT was used for attenuation correction and anatomical localization of the active BAT. The scans were analyzed with PMOD software (version 3.0; PMOD Technologies). The regions of interest were manually outlined, while a threshold of 1.5 SUV (standardized uptake value) and Hounsfield units between -10 and -180 were used, as described previously by our group (23). Additionally fixed volumes (10mm by 10 mm) were placed in the

cervical adipose tissue behind the clavícula to measure general uptake values as described before (24). BAT activity was expressed in SUV ([¹⁸F]-FDG uptake (kBq/mL/ (injected dose (kBq/patient weight (g))). The activity was determined as average SUV (SUV_{mean}) and as total SUV (SUV_{mean} times the volume of interest).

Statistics

Two means were compared using student's t-test, or with the Wilcoxon-signed rank test in case of non-parametric data. Comparison of multiple means was assessed by ANOVA. $p < 0.05$ was considered statistically significant. Analyses were performed using Graph Pad Prism (San Diego, CA).

RESULTS

Thermogenesis is enhanced in mice fed a HFD supplemented with NR

NR has been shown to have metabolic effects especially in metabolically challenged animal models. Therefore, to examine BAT metabolism following NR supplementation, we utilized an *in vivo* mouse model of early insulin resistance and obesity induced by HFD feeding (25). Thus, C57BL/6J mice were fed a HFD for 4 weeks supplemented with or without NR at a concentration of 400 mg/kg/day. NR supplementation decreased BAT mass compared to control (**Figure 1A**), indicative of increased BAT activity. Next, animals were challenged with cold (4 °C) for 6 hrs. Animals on a HFD showed a significant drop in body temperature following acute cold exposure; however, this decrease in body temperature was prevented by supplementation of NR to HFD (**Figure 1B**).

NR does not affect *ex vivo* lipolysis in murine BAT

In order to examine whether NR supplementation affects lipolysis in BAT in mouse, we performed *ex vivo* lipolysis experiments using mouse BAT harvested at room temperature (21 °C) and after acute cold exposure (4 °C). At room temperature, supplementation of NR to a HFD did not alter lipolysis (**Figure 1C**). Also, under conditions of acute cold exposure, supplementation of NR did not significantly change lipolysis in BAT (**Figure 1C**). UCP1 protein abundance remained unaltered following NR supplementation. Also, the addition of acute cold did not change UCP1 protein expression in mouse BAT (**Figure 1D**).

Figure 1. NR stimulates BAT metabolism in mice fed a high fat diet. Mice were fed a high fed diet (HFD) or a high fat diet supplemented with NR (HFD+NR). Mice were kept at room temperature (RT) or acutely cold exposed for six hours (cold). **A.** BAT mass was weighed at the end of intervention. **B.** Body temperature was measured for six hours during cold exposure in mice fed a high fat diet (HFD) or mice fed a high fat diet supplemented with NR (HFD+NR). **C.** *Ex vivo* lipolysis was performed on BAT explants. **D.** UCP1 protein in mouse BAT. Data is expressed as mean $\pm$ SE. * $p < 0.05$ ($n = 5-7$).

NR combined with cold exposure decreases lipid droplet size in murine BAT

Based on our finding that NR decreased BAT mass, we next sectioned BAT and stained it with HE in order to get more understanding of BAT morphology and adipocyte size. Brown adipocyte cell size decreased following NR supplementation to HFD (**Figure 2A and B**). Likewise, acute cold exposure decreased adipocyte cell size. NR supplementation and cold combined resulted in the smallest adipocyte cell size (**Figure 2A and B**), indicating active BAT via increase lipolysis. In order to test the involvement of UCP1, we examined UCP1 via immunofluorescence in murine BAT by, which was unaltered following NR supplementation or cold treatment (**Figure 2C**) matching the data obtained by western blot analysis of protein expression (**Figure 1D**). Also, mitochondrial density examined by TOMM20 immunofluorescence was unchanged after NR supplementation or cold stimulation (**Figure 2D**).

Figure 2. NR supplementation alters adipocyte morphology in mouse BAT following a high fat diet. Mice were fed a high fat diet (HFD) or a high fat diet supplemented with NR (HFD+NR). Following the four-week diet, animals were acutely exposed to cold (cold) or remained at room temperature (RT). **A.** HE staining of BAT. **B.** Cell size quantification of results obtained in panel A. **C.** Immunofluorescent staining of UCP1 in mouse BAT (green). Nuclei were stained with DAPI (blue). **D.** Immunofluorescent staining of TOMM20 in mouse BAT (green). Nuclei were stained with DAPI (blue). Data is expressed as mean $\pm$ SE ($n=2-3$).

NR stimulates NE-stimulated mitochondrial uncoupling only in adipocytes derived from human BAT

To investigate if the positive effects of NR on BAT activity in mouse can be translated to humans, we incubated cultured adipocytes derived from human BAT and WAT with 50 or 500 μM NR to examine effects on NE-induced mitochondrial uncoupling. Whereas 50 μM NR did not increase NAD^+ levels, 500 μM NR significantly increased NAD^+ levels in adipocytes derived from human BAT and WAT (**Figure 3A and B**). Both doses of NR were without effect on ATP concentrations in adipocytes derived from human BAT or WAT (**Figure 3C and D**). To further investigate the effect on mitochondrial uncoupling in adipocytes derived from BAT and WAT, cultured adipocytes were treated with oligomycin to block ATPase and subsequently

Figure 3. NR stimulates mitochondrial uncoupling in cultured primary adipocytes derived from human BAT. NAD⁺ (A, B) and ATP (C, D) levels were determined in cultured adipocytes derived from human BAT or WAT after 24 hours with 0 μ M, 50 μ M or 500 μ M NR. Data is expressed as a fold increase compared to 0 μ M NR. Cellular respiration was measured using the Seahorse bioanalyzer in cultured adipocytes derived from human BAT (E) and WAT (F) following 24 hour incubation with 0 μ M, 50 μ M or 500 μ M NR. Mitochondrial respiration was measured following injections with oligomycin, NE and FCCP. G. Quantification of AUC from results in panel E. H. Quantification of AUC from results in panel F. NR does not alter lipolysis in cultured adipocytes derived from human BAT. Cultured adipocytes derived from human BAT (I) and WAT (J) were incubated for 24 hours with 0 μ M, 50 μ M or 500 μ M NR. Following adipocytes were stimulated with NE in order to measure glycerol release as a marker of lipolysis. Data is expressed as mean $\pm$ SE. * p <0.05 (n=4-6)

stimulated with NE. NE enhanced mitochondrial uncoupling in adipocytes derived from BAT, while this response was negligible in adipocytes derived from human WAT (**Figure 3E and F**). 50 μ M and 500 μ M NR were unable to potentiate NE-stimulated mitochondrial uncoupling in WAT (**Figure 3F and H**). In human primary brown adipocytes however, 500 μ M NR increased NE-stimulated mitochondrial uncoupling (**Figure 3E and G**), as well as maximal FCCP-induced mitochondrial respiration (**Figure 3E and G**). At 500 μ M NR, antimycin A and rotenone resulted in higher OCR compared to 0 μ M NR and 50 μ M NR.

NR does not affect lipolysis in cultured adipocytes derived from human BAT and WAT

To examine if the effect of NR on mitochondrial uncoupling in human BAT cells was mediated via elevated lipolysis, we examined lipolysis following NR treatment in cultured adipocytes derived from human BAT and WAT. Both basal and NE-stimulated lipolysis was unchanged following NR treatment in adipocytes derived from human BAT and WAT (**Figure 3I and J**).

NR does not change gene expression in adipocytes derived from human BAT

SIRT6s are NAD⁺-dependent deacetylases and NR stimulates SIRT6 activity by increasing the level of its obligatory co-substrate NAD⁺ (26). Next we assessed whether NR was able to alter gene expression of SIRT6-related pathways. Adipocytes derived from human BAT and WAT were incubated with vehicle, 50 μ M NR or 500 μ M NR. In cultured adipocytes derived from human WAT, 500 μ M NR increased transcript levels of Tfam compared to vehicle (**Figure 4F**). In cultured adipocytes derived from human BAT, NR did not significantly change the transcript levels of any of the genes assessed (UCP1, SIRT6, SOD2, PGC1A or TFAM; **Figure 4A-F**). We also determined the levels of OXPHOS proteins by western blot examining the individual complexes in adipocytes derived from human BAT and WAT. The highest concentration of 500 μ M NR decreased the amount of complex I (**Figure 4G**) in adipocytes derived from human BAT, while 50 μ M NR increased protein abundance of complex I in adipocytes derived from human WAT (**Figure 4H**).

Figure 4. NR does not alter gene expression of SIRT targets in cultured primary adipocytes derived from human BAT. Cultured adipocytes derived from human BAT (black bars) and WAT (white bars) were stimulated for 24 hours with 0 μ M (0), 50 μ M (50) or 500 μ M (500) NR. Gene expression of UCP1 (A), SIRT3 (B), SOD2 (C), SIRT1 (D), PGC1a (E) and TFAM (F) were determined using qPCR techniques. Cultured adipocytes derived from human BAT (G) and WAT (H) were stimulated for 24 hours with 0 μ M (0), 50 μ M (50) or 500 μ M (500) NR. OXPHOS complexes were analyzed by Western blot. Data is expressed as mean $\pm$ SE. * p <0.05 compared to matching control (n=4).

NR does not affect BAT activity or energy expenditure in human volunteers

Our results show that NR can enhance BAT activity in mice and human primary brown adipocytes. These promising results urged us to examine the true translational potential of NR on human BAT and therefore we performed a first double-blind placebo-controlled cross-over design clinical trial in a small cohort of obese volunteers. During six weeks, 1000 mg/day NR or placebo was supplemented. Cold-stimulated BAT activity was determined via uptake of [18F]-FDG using PET-CT and whole-body energy expenditure was also measured. However, NR supplementation for six weeks had no effect on [18F]-FDG mediated glucose uptake accessed as

SUVmean and SUVmax in fixed volumes (**Figure 5A and B**). NR supplementation was also unable to increase cold-stimulated energy expenditure (**Figure 5C and D**). Also, non-shivering thermogenesis was unchanged following NR supplementation (**Figure 5E**).

Figure 5. NR does not stimulate energy expenditure or BAT activity in humans. Human volunteers were supplemented with 1000 mg/day NR for six weeks in a placebo-controlled cross over design. BAT SUV mean (**A**) and BAT SUV max in fixed volumes (**B**) following cold exposure. Energy expenditure at thermoneutrality and after cold exposure following placebo (**C**) or NR (**D**). Non-shivering thermogenesis (NST) following NR supplementation (**E**). Data is expressed as mean $\pm$ SE. * $p < 0.05$ ($n = 9$)

DISCUSSION

Preclinical data has demonstrated that increasing NAD⁺ levels can counteract harmful effects of metabolic disease, and NR supplementation is a powerful intervention to do so. Here, we specifically assessed the effect of NR, on mouse BAT *in vivo* and on adipocytes derived from human BAT and WAT *in vitro* and *in vivo* in human volunteers. In HFD-fed mice, NR supplementation resulted in enhanced tolerance to cold and decreased lipid droplet size. Similarly, in cultured adipocytes derived from human BAT, elevating NAD⁺ levels via NR enhanced NE-mediated mitochondrial uncoupling. This stimulatory effect was specific for adipocytes derived from human BAT, which is in line with earlier observation in animals where NAD⁺ supplementation increased NAD⁺ concentrations in BAT and stimulated BAT metabolism following a cold challenge (11). However, six weeks of supplementation with 1000 mg/day NR did not increase BAT activity in humans. These observations together show that NR and beta-adrenergic signaling can work together to enhance BAT activity, however the current dose of NR in humans was unable to increase human BAT activity *in vivo*.

We examined lipolysis *ex vivo* in murine BAT and *in vitro* in human BAT in the context of NR stimulation. There are obvious differences when comparing murine adipose tissue explants to *in vitro* differentiated human adipocytes. However, *in vitro* lipolysis in adipocytes derived from human BAT was unaltered following NR and also *ex vivo* lipolysis in mouse BAT following NR exposure was unaffected. This indicates that the direct mode of action of NR is most likely independent of lipolysis in mice and humans. Previously, it has been reported that NR increases NAD⁺ content in cells and mitochondria (11) and mitochondria are pivotal players in providing energy for lipolysis. In line with unaltered lipolysis in human and murine BAT, we also observed no change in OXPHOS protein, ATP levels or gene expression related to mitochondrial biogenesis in cultured adipocytes derived from human BAT. This was further substantiated with results from TOMM20 staining in mouse BAT which were unchanged following NR treatment. Also, the protein or mRNA abundance of the classical mitochondrial marker for BAT, UCP1, was unchanged following NR treatment in mouse BAT and in cultured adipocytes derived from human BAT, respectively. However, when it comes to UCP1 expression and NR, different results have been obtained depending on the experimental setup. In young lean mice on a chow diet supplemented for five weeks with NR, UCP1 protein content was increased in BAT, however, in this study total BAT mass was not reported (27). In another study, NR was administered directly after birth in mice which was subsequently followed by a HFD-challenge for ten weeks. Ucp1 mRNA expression was unaffected in animals on a HFD in BAT, but interestingly the animals on a control diet showed decreased

expression of Ucp1 mRNA expression following NR in BAT (28). These findings together indicate that gene and/or protein expression alone might not be the best indicator for BAT metabolic activity.

Stimulating BAT activity by other means than cold in humans could have beneficial effects on whole body metabolism. Human BAT activity shows a negative correlation with obesity (4, 29), and stimulation of BAT activity through cold acclimatization increases energy expenditure that potentially could lead to loss of fat mass (30). Furthermore, cold-induced BAT activity is related to increased insulin sensitivity in humans (31, 32). Cold exposure is the most effective method to stimulate BAT activity; however, not the most practical one. Therefore, alternative routes to stimulate BAT in humans are warranted. To test if NR may have potential to activate BAT in humans, an *in vitro* model for human BAT activity is a good tool that can provide insights into human metabolism. Previously, we demonstrated that bile acid supplementation increased mitochondrial uncoupling in adipocytes derived from human BAT, a finding we also confirmed in humans (8). Therefore, we believe that primary cultured adipocytes derived from human BAT are a good model to investigate compounds that activate human BAT. Furthermore, we have demonstrated that *in vitro* mitochondrial uncoupling in adipocytes derived from human BAT correlates with BMI and age, a finding that is also observed in human BAT activity *in vivo* (29). All these observations together show that an *in vitro* model can aid in providing information on *in vivo* metabolism of human BAT. In the current study, NR was able to stimulate BAT activity in human primary brown adipocytes. However, we could not confirm these results in a clinical trial in humans. The reason why NR had beneficial effects in human primary brown adipocytes but not *in vivo* in humans, cannot be deduced from the current study. Different dosage or duration of NR supplementation may be needed to exert beneficial effects of NR on human BAT *in vivo*. Therefore, further translational research is needed in order to better understand the differences in NAD⁺ metabolism between mouse and man.

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EBMN MM CR KG JAJ GS JA WDvML PS designed the study. EBMN MM CR KG JAJ GS performed experiments. EBMN MM CR KG JA WDvMK PS analyzed and interpreted the data. EBMN MM CR KG JA WDvMK PS contributed to the discussion and wrote, reviewed and edited the manuscript. All authors approve the last version of the manuscript. This research was funded by CVON ENERGISE grant CVON2014-02 to PS. JA is supported by École Polytechnique Fédérale de Lausanne (EPFL), the Velux Stiftung (1019), and the Swiss National Science Foundation (31003A_179435). The authors would like to thank Hongbo Zang for experimental assistance and reading the manuscript. PS 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. The authors declare no conflict of interest relevant to this article.

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CHAPTER 4

Non-shivering cold acclimation does not improve postprandial glucose and lipid metabolism or insulin sensitivity in patients with type 2 diabetes

Carlijn M.E. Remie*, Michiel P.B. Moonen*, Kay H.M. Roumans, Bas Havekes, Anne Gemmink, Emmani B.M. Nascimento, Gert Schaart, Esther Kornips, Peter J. Joris, Vera B. Schrauwen-Hinderling, Joris Hoeks, Sander Kersten, Matthijs K.C. Hesselink, Esther Phielix, Wouter D. van Marken Lichtenbelt, Patrick Schrauwen

* These authors contributed equally

In preparation

ABSTRACT

Background & Aims: Type 2 diabetes is a major health problem and is associated with increased cardiovascular disease risk. Cold exposure has received much attention over the past years as a potential strategy to improve metabolic health. We previously showed that ten days of cold acclimation with non-shivering thermogenesis (NST) markedly improved skeletal muscle insulin sensitivity in patients with type 2 diabetes. However, whether cold acclimation with NST also affects postprandial metabolism and cardiovascular risk markers remains unknown. Here, we aimed to investigate whether cold acclimation with NST beneficially affected postprandial metabolism and cardiovascular risk markers, and if the effect on insulin sensitivity was sustained for a longer period of time. In addition, we hypothesized that beneficial effects of cold exposure could be achieved by stimulating NST, hence paid special attention to the prevention of shivering in our participants.

Methods: Nine obese participants diagnosed with non-insulin dependent type 2 diabetes were exposed to mild cold (16-17 °C) for ten days. Before and immediately after the cold exposure postprandial glucose and lipid metabolism, vascular function measurements, insulin sensitivity and intrahepatic lipid content were determined. Furthermore, skeletal muscle biopsies were obtained. Lastly, insulin sensitivity was measured again ten days later after the last cold exposure session to assess the sustained effect of cold acclimation.

Results: Based on direct observations, questionnaire results and gene expression analyses in muscle biopsies, no shivering occurred during the ten-day cold acclimation. Ten-day cold acclimation with NST significantly increased postprandial energy expenditure (AUC pre: 2620 ± 146 ; post: 2752 ± 168 kJ, $p=0.03$). In addition, overnight fasted carbohydrate oxidation was significantly increased (pre 3.76 ± 0.47 vs. post 4.97 ± 0.68 $\mu\text{mol/kg/min}$, $p<0.01$) and fat oxidation significantly decreased after the cold acclimation (pre 3.80 ± 0.17 vs. post 3.57 ± 0.17 $\mu\text{mol/kg/min}$, $p<0.01$). Aortic augmentation index was significantly improved after cold acclimation measured in the overnight fasted state (pre 22.57 ± 1.36 % vs. post 19.84 ± 1.96 %, $p=0.03$). Cold acclimation with NST had no effect on postprandial glucose and lipid metabolism, intrahepatic lipid content or other vascular function markers. Surprisingly, insulin sensitivity was unaffected both when measured directly (one day) or ten days after the last cold exposure.

Conclusion: A ten-day cold acclimation period at 16-17 °C under non-shivering conditions resulted in mild effects on substrate oxidation and cardiovascular risk markers. The lack of marked effects in the current study may be attributed to the absence of shivering compared to previous studies. This suggests that shivering is an essential component of the metabolic health effects induced by cold exposure.

INTRODUCTION

Mortality rates in type 2 diabetes mellitus (T2DM) patients are approximately twice as high compared to individuals without T2DM and can mostly be attributed to an increased risk of coronary heart diseases (1, 2). In the last decade, cold exposure as a tool to alleviate insulin resistance has attracted lots of scientific interest. Currently, there is little demand for the human body to adjust to colder temperatures, since humans spend most of their time in a well-controlled indoor environment with optimal temperatures within the body's thermoneutral zone. The physiological reaction of the human body to cold exposure includes simultaneously 1) insulative responses by peripheral vasoconstriction and 2) an increase in metabolic rate by shivering thermogenesis (ST) and/or non-shivering thermogenesis (NST). It has been shown that during daily cold exposure in humans, shivering gradually decreases within 10-20 days, while the related increase in metabolic rate remains at a stable level (3-5). This indicates that acclimation to cold occurs and that ST can be replaced by NST. Both brown adipose tissue (BAT) (6-9) and skeletal muscle (4, 10) have been identified as contributors to NST.

Cold acclimation has been shown to promote insulin sensitivity in humans, originally attributed to increased BAT activity (11, 12). Research performed within our group, however, has shown that ten days of cold acclimation (14-15 °C) markedly improved skeletal muscle insulin sensitivity by 43% in patients with type 2 diabetes (13), an improvement that is comparable to the effect seen after long term exercise training (14). Interestingly, the cold induced improvement of insulin sensitivity did not appear to originate from BAT activation but was associated with increased GLUT4 translocation in skeletal muscle (13). Therefore, these findings indicate that effects of cold acclimation on skeletal muscle may be a novel approach to improve metabolic health in patients with T2DM.

Hyperglycemia and dyslipidemia are common metabolic abnormalities in patients with T2DM (15), that most commonly are alleviated upon insulin sensitizing interventions. Interestingly, animal studies have shown reduced postprandial lipids upon prolonged cold exposure (16). Moreover, a postprandial reduction in hypertriglyceridemia in human individuals was associated with BAT (17). Lowering postprandial hyperglycemia and hypertriglyceridemia is clinically relevant as high plasma glucose and triglyceride levels can cause damage to the vascular wall inducing an impaired vascular function (18), which causes atherosclerotic plaque development that may ultimately lead cardiovascular disease events. However, the

effect of cold exposure on these cardiovascular risk markers has not yet been investigated in humans.

Therefore, we primarily aimed to investigate the effect of ten days cold acclimation with NST in overweight and obese patients with T2DM on postprandial glucose and lipid metabolism and cardiovascular risk markers. Secondly, we investigated whether a ten-day cold acclimation period with NST affects insulin sensitivity, and if so, if this effect is sustained for another ten days at room temperature. Given the previous results on skeletal muscle (13), we also aimed to test the hypothesis that beneficial effects of cold exposure can be truly achieved by stimulating NST, and hence took specific care to prevent shivering in our participants.

METHODS

Participants

Ten obese men and women were included in the study with one volunteer dropping out because of personal reasons. Hence, nine participants completed the study. All participants underwent a screening including assessment of blood parameters, electrocardiography, anthropometric measurements and a questionnaire to evaluate eligibility. Inclusion criteria were: 45-70 years of age, BMI 27-35 kg/m², diagnosed with type 2 diabetes for at least one year (relatively well-controlled HbA1c <8.5%), use of oral glucose lowering drugs (metformin and/or sulfonylurea agents), a sedentary lifestyle (<3 h exercise per week), non-smoking for at least six months, no alcohol use of >2 servings per day and a stable body weight for at least six months. Other medication without known effects on the primary outcome measurement was allowed. Data were collected between March 2016 and August 2017.

Ethical approval

The study was conducted according to the declaration of Helsinki and was approved by the Ethics Committee of the Maastricht University Medical Center. The study was registered at <https://trialregister.nl> (NL4469/NTR5711). All participants provided their written informed consent before screening.

Study design

Figure 1 provides an overview of the study design. A ten-day cold acclimation intervention was performed essentially as previously described (13), although the environmental temperature was set approximately 1-2 °C higher in the current study,

at 16-17 °C, to avoid shivering. Before the onset of the cold acclimation period a high-fat meal test (meal test pre) was combined with several vascular function measurements. Three days later, intrahepatic lipid content was measured followed by a muscle biopsy and a hyperinsulinemic-euglycemic 2-step clamp (clamp pre). Subsequently, a ten-day cold acclimation intervention started (Day 1-10). One day after the last cold exposure, (Day 11), the first test day was repeated (meal test post). The following two days (Day 12-13), additional cold exposure was maintained in order to sustain the intervention effect for the re-tests on Day 14 (clamp post). Ten days after termination of the cold acclimation, i.e. without further intervention, another muscle biopsy was taken followed by a hyperinsulinemic-euglycemic 2-step clamp (clamp long term) to assess the potential long-term effect of cold acclimation on whole body insulin sensitivity. Two months after the last test day, fasting blood samples were taken to measure markers for glucose homeostasis. All tests were executed at room temperature. In the evening preceding all test days, participants consumed a standardized meal and remained fasted from 20:00 h onwards. In addition, participants were asked to refrain for at least 48 hours from any physical activity different from their daily routine. Participants arrived at the testing facility by car. Blood glucose lowering medication was discontinued on the morning of the clamps.

Figure 1. Study design

A meal test and clamp were performed before cold acclimation, separated by two days. After ten days of cold exposure a second meal test was performed, followed by two additional days of cold exposure and followed by a second clamp. Ten days after the last intervention day, a third clamp was performed. Two months after the last clamp another blood sample was collected. Blue boxes represent cold acclimation intervention (16-17 °C). White boxes represent no intervention. Black boxes represent test days.

Cold acclimation

During cold acclimation, participants were progressively exposed to an environmental temperature of 16-17 °C for ten consecutive days: two hours on day 1, four hours on day 2 and six hours on days 3 to 10. On Day 11-12, after the first post-intervention measurements, participants were again cold exposed for six hours. Participants were dressed in short-sleeved T-shirts and shorts and remained sedentary while staying in the cold room and were instructed to not change their habitual diet during the entire

study period. Food intake while staying in the cold room was kept constant. Wireless temperature sensors (iButtons, Maxim Integrated, San Jose, CA, USA) were placed on 15 ISO-defined sites on days 3 and 10 of the cold acclimation period to measure skin temperature. Average skin temperature as well as proximal and distal temperature was calculated as described before (19). Participants were not allowed to shiver. In case shivering started, extra clothing was immediately provided to ensure that shivering stopped. At selected timepoints during day 3 and 10 VAS scales on thermal sensation, thermal comfort and shivering were completed. Incremental AUCs (iAUC) were calculated to determine subjective responses during the cold acclimation period, as described before (8).

High-fat meal test

After placing an intravenous cannula, a fasted blood sample was drawn ($T=0$) and energy expenditure and substrate oxidation was measured via indirect calorimetry. At 09:00 h, participants were asked to consume a high-fat shake, within ten minutes. The nutritional content of the shake is shown in **Supplementary Table 2**. Subsequently, blood samples were drawn after 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420 and 480 minutes. After the blood sampling at $T=240$, at 13:00 h, the participants consumed a second high-fat shake with the exact same contents as in the morning to investigate the second-meal effect, as described before (20). At T_{30-60} , T_{90-120} , $T_{210-240}$, $T_{270-300}$, $T_{350-380}$ and $T_{450-480}$ indirect calorimetry was performed to measure energy expenditure and substrate oxidation. Participants were not allowed to eat or drink anything else throughout the meal test, except for water.

Vascular function measurements

On the morning of the meal tests, radial artery pulse wave analysis (PWA) was performed with a tonometer (SphygmoCor v9, AtCor Medical, West Ryde, Australia), from which the aortic augmentation index corrected for heart rate (AIx_{HR75}) was calculated as described before (21). Using the same tonometer, carotid-to-femoral pulse wave velocity (PWV_{c-f}) was also determined as described before (21). These vascular stiffness measurements were performed before (T_0) and during the high-fat meal tests at two different time points (T_{120} and T_{300}).

Retinal vascular images were obtained to measure microvascular effects of the cold acclimation. Retinal images were obtained as described before using a nonmydriatic retinal camera (Topcon TRC-NW-300; Topcon Co.) (22). At least two arteriolar and two venular segments were measured and summarized by using the Parr-Hubbard formulas (23), special care was taken to examine the exact same segments before and after the cold acclimation period.

Hyperinsulinemic-euglycemic clamp

To determine insulin sensitivity, a two-step hyperinsulinemic euglycemic clamp (24) with co-infusion of D-[6,6-²H₂] glucose tracer (0.04 mg/kg/min) was performed, as described before (25). In short, insulin-suppressed endogenous glucose production (EGP) during low insulin infusion (10 mU/m²/min) was measured as a reflection of hepatic insulin sensitivity. This was followed by a high insulin infusion (40 mU/m²/min) to measure whole body glucose disposal (Rd). Indirect calorimetry was performed during baseline, low insulin and high insulin to measure energy expenditure and substrate oxidation. Steele's single pool non-steady state equations were used to calculate glucose appearance (Ra) and glucose disposal (Rd) (26). Volume of distribution was assumed to be 0.160 l/kg for glucose. Whole-body insulin sensitivity (S_i) was calculated according to Bergman et al. (27), taking differences in insulin and glucose levels into account: $S_i = \Delta Rd / (\Delta insulin * clamping \text{ glucose})$, where Δ represents the change from the basal state to the insulin-stimulated condition. EGP was calculated as Ra minus exogenous glucose infusion rate. Non-oxidative glucose disposal (NOGD) was calculated as Rd minus carbohydrate oxidation.

Indirect calorimetry

Whole body oxygen consumption and carbon dioxide production were measured during specific timepoints in the meal tests and the clamps using an automated respiratory gas analyzer with a ventilated hood system (Omnical; Maastricht Instruments, Maastricht, The Netherlands). Participants were measured in supine position for 30 minutes each time. Energy expenditure, glucose oxidation and fat oxidation rates were calculated using equations based on the measured averaged oxygen and carbon dioxide concentrations with the assumption that protein oxidation was negligible (28, 29).

Intrahepatic lipid quantification by MR spectroscopy

Proton magnetic resonance spectroscopy (¹H-MRS) was used to quantify intrahepatic lipid content (IHL) at 07:00 h in the morning of Clamp pre and Clamp post. All measurements were performed on a 3.0T whole body scanner (Achieva Tx, Philips Healthcare, Best, The Netherlands). Spectra were acquired as described before (30). Values are given as T2 corrected ratios of the CH₂ peak relative to the unsuppressed water peak, expressed as percentage.

Skeletal muscle biopsies

On each clamp day, a muscle biopsy was taken from the m. vastus lateralis under local anesthesia (2% lidocaine, without epinephrine) using the Bergström technique (31). The muscle biopsy was taken before the start of the insulin infusion on the

morning of the clamp, at 08:30 h after an overnight fast. The biopsy immediately frozen in melting isopentane cooled with liquid nitrogen for biochemical analyses and immunohistochemical analyses.

Histochemical analysis of GLUT4 in skeletal muscle biopsies

Muscle biopsies taken in the overnight fasted state, prior to all three clamps, were analyzed for GLUT4 translocation. Sections were stained for GLUT4 as described previously (13), with primary antibodies against GLUT4 (sc-1608, Santa Cruz, Dallas, USA) and Laminin (L-9393, Sigma, St. Louis, USA), and appropriate conjugated secondary antibodies AlexaFluor488 (AF488, Invitrogen, Life Technologies Europe, Bleiswijk, The Netherlands) and AlexaFluor555 (AF555, Invitrogen, Life Technologies Europe). Images were acquired with a Nikon E800 fluorescent microscopy (Nikon, Amsterdam, The Netherlands) as described previously (13). Images were analyzed using ImageJ (NIH, Bethesda, USA) (32). Mean intensity of GLUT4 was measured on the cell membranes and in the cytosol.

Shivering markers in skeletal muscle biopsies

RNA extraction and cDNA synthesis from skeletal muscle was performed as previously described (33, 34). In short, RNA was isolated from skeletal muscle biopsies using Trizol followed by purification using the RNeasy kit from Qiagen (Hildenberg, Germany). cDNA was created by using the high-capacity RNA-to-cDNA kit from Applied Biosystems (Foster City, USA). mRNA expression was determined using a CFX384 Touch Real-Time PCR Detection System from BioRad Laboratories (Hercules, CA) using the following Taqman assays: CHRNA1 (Hs00909664), CCDC39 (Hs00977326), MYH8 (Hs00267293), CCDC80 (Hs00277341), CHRND (Hs00897937), LYRM9 (Hs00953770), ACTC1 (Hs01109515) and RRAD (Hs00188163). Gene expression was normalized to RPLPO (fwd: CCATTCTATCATCAACGGGTACAA, rev: AGCAAGTGGAAGGTGTAATCC) and expression was analyzed via the standard curve method.

Blood sampling and analyses

Blood collected in EDTA-coated tubes was immediately stored on ice, centrifuged and plasma was stored at -80 °C until further analyses. Blood collected in serum-tubes was stored at room temperature for at least 30 minutes to allow coagulation, followed by centrifugation and storage at -80 °C until further analyses. Glucose (Hk-CP, Axonlab, Amsterdam, The Netherlands) and FFA (NEFA-HR, WAKO chemicals, Neuss, Germany) were determined enzymatically in EDTA plasma derived from the meal test samples using a Pentra 400 (Horiba, Montpellier, France). Insulin levels

during the meal test were analyzed using RIA, and insulin levels during the clamp were analyzed using ELISA. Triglycerides (Sigma, Zwijndrecht, The Netherlands), cholesterol (CHOD-PAP, Roche Diagnostics, Mannheim, Germany) and HDL-cholesterol (CHOD-PAP, Roche Diagnostics, Mannheim, Germany) after precipitation of apoB-containing lipoproteins with phosphotungstic acid and magnesium ions, were analyzed in serum also using a Pentra 400. LDL-cholesterol was calculated according the Friedewald equation (35).

Statistical analyses

Participant characteristics are reported as mean $\pm$ SD. Other results are reported as mean $\pm$ SE. Data are presented for $n=9$, unless otherwise indicated. Differences between Clamp pre, Clamp post and Clamp longterm were analyzed with a nonparametric Friedman test. Posthoc analyses were performed with Dunn's multiple comparison test. Differences between Meal test pre and Meal test post were analyzed with a Wilcoxon matched pairs signed rank test. Differences between muscle gene expression in the current and previous study were analyzed with a Mann-Whitney test. Statistical significance was set at $p<0.05$. Statistical analyses were performed using IBM SPSS version 23.0 for MacOSx (IBM, Armonk, NY, USA).

RESULTS

Participant characteristics

Nine overweight to obese men and women (age 65 ± 5 years; BMI 32.1 ± 2.8 kg/m²; four women) participated in the study (see **Table 1**). As defined by the inclusion criteria, participants were diagnosed for at least one year with type 2 diabetes and were treated with oral medication only (see **Supplementary Table 1**). Participants were non-smokers, had no other active diseases and had a sedentary lifestyle according to the Baecke questionnaire score (7.94 ± 2.00 , **Table 1**).

Body and room temperature, thermal comfort and shivering during cold acclimation

Room temperature during cold acclimation was on average 16.4 ± 0.30 °C, which was approximately 1.4 °C higher compared to the previous study by Hanssen et al (13). Average skin temperature dropped from 27.5 ± 0.33 °C to 26.3 ± 0.97 °C during day 3. This was similar to the drop in average skin temperature from 27.4 ± 0.80 °C to 26.4 ± 1.26 °C on day 10. Thermal sensation and thermal comfort, assessed via VAS scales, was not significantly different between day 3 and day 10 of cold acclimation, as shown in **Supplementary Figure 1**. The temperature was progressively perceived colder and more uncomfortable over time. The shivering questionnaires

revealed that participants experienced no shivering and only occasionally reported tense muscles. Self-reported shivering intensity was less than described in the study from Hanssen et al (13) and were not significantly different between day 3 and day 10 of cold acclimation (**Figure 6A**).

Table 1. Participant characteristics

Parameter	Mean $\pm$ SD
Gender F/M	4/5
Age (years)	65 $\pm$ 5
Body weight (kg)	93.7 $\pm$ 17.3
Height (m)	1.70 $\pm$ 0.10
BMI (kg/m ²)	32.1 $\pm$ 2.8
HbA1c (%)	7.3 $\pm$ 0.7
TG (mmol/L)	1.54 $\pm$ 0.34
ASAT (U/L)	24 $\pm$ 8
ALAT (U/L)	37 $\pm$ 19
GGT (U/L)	34 $\pm$ 13
eGFR (ml/min/1.73m ²)	79 $\pm$ 9
Physical activity level (Baecke score)	7.51 $\pm$ 1.16

Abbreviations: BMI, body mass index; HbA1c, hemoglobin A1c; TG, triglycerides; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; GGT, gamma-glutamyl transferase; eGFR, estimated glomerular filtration rate according CKD-EPI method.

Postprandial metabolism and substrate kinetics

Meal test total area-under-the-curve values (AUC) for glucose, insulin and triglycerides were not significantly different before and after cold acclimation ($p=0.43$, $p=0.65$ and $p=0.50$ respectively, **Figure 2A-C** and **Supplementary Table 4**). In addition, no significant differences were observed in AUC when the 1st or 2nd meal of the meal test were analyzed separately (**Supplementary Table 4**). Area under the curve for plasma free fatty acids was also not significantly different before and after ten days of cold acclimation ($p=0.16$, **Figure 2D** and **Supplementary Table 4**). However, when the 1st and 2nd meal of the meal test were analyzed separately, the total AUC for FFA during the 1st meal was significantly lower after cold acclimation (pre: 94228 ± 5115 mmol/l; post: 86014 ± 4545 mmol/l, $p=0.039$) as shown in **Supplementary Table 4**.

Total energy expenditure during the meal tests, calculated as AUC, was significantly higher after the cold acclimation (pre: 2620 ± 146 ; post: 2752 ± 168 kJ, $p=0.03$, **Figure 3A** and **Supplementary Table 4**). Although this elevated energy expenditure seemed

to be mainly due to higher glucose oxidation, the differences in glucose and fat oxidation during the meal tests were not statistically significant ($p=0.44$ and $p>0.99$ respectively, **Figure 3B-C, Supplementary Table 4**).

Figure 2. Plasma levels during the meal tests

A: plasma levels of glucose presented, **B:** Insulin, **C:** Triglycerides and **D:** Free fatty acids during the meal test. Pre cold acclimation is presented as the red line, post cold acclimation as the blue line. Dashed vertical lines indicate the time of consumption of the 1st shake at T0 and the 2nd shake at T240. $n=9$. Data is presented as mean $\pm$ SE.

The results of the meal test suggest that cold acclimation may increase energy expenditure, probably fueled by higher carbohydrate oxidation. To further test this, we analyzed energy expenditure and substrate oxidation measured in the morning of the clamps. Overnight fasted energy expenditure at the start of the clamp (baseline) was not affected by cold acclimation (**Supplementary Table 3**). However, energy expenditure during the high insulin phase was significantly higher after cold acclimation compared to before (pre 4.67 ± 0.30 vs. post 4.89 ± 0.27 kJ/min, $p=0.03$) and this elevation tended to return to pre-intervention levels on the longterm (post 4.89 ± 0.27 vs. long term 4.67 ± 0.18 kJ/min, $p=0.10$, **Supplementary Table 3**). Carbohydrate oxidation after an overnight fast was significantly higher after ten days

of cold acclimation compared to before (pre 3.76 ± 0.47 vs. post 4.97 ± 0.68 $\mu\text{mol/kg/min}$, $p < 0.01$, **Supplementary Table 3**). In addition, fat oxidation after an overnight fast was significantly lower directly after cold acclimation compared to before (pre 3.80 ± 0.17 vs. post 3.57 ± 0.17 $\mu\text{mol/kg/min}$, $p < 0.01$, **Supplementary Table 3**). These effects on carbohydrate and fat oxidation did not sustain on the long term (**Supplementary Table 3**). The changes in substrate oxidation after cold acclimation were not observed during insulin infusion (**Supplementary Table 3**).

There was no significant difference after cold acclimation for baseline HDL (pre 1.12 ± 0.12 vs. post 1.21 ± 0.08 mmol/l , $p = 0.16$), LDL (pre 1.80 ± 0.24 vs. post 1.84 ± 0.24 mmol/l , $p = 0.82$) or total cholesterol (pre 3.57 ± 0.31 vs. post 3.76 ± 0.31 mmol/l , $p = 0.16$).

Figure 3. Energy expenditure and substrate oxidation during the meal tests

A: Energy expenditure, **B:** Carbohydrate oxidation (CHox), and **C:** Fatty acid oxidation (FAox) during the meal test. Pre cold acclimation is presented as the red line, post cold acclimation as the blue line. Dashed vertical lines indicate the time of consumption of the 1st shake at T0 and the 2nd shake at T240. $n = 7$. Data is presented as mean $\pm$ SE.

Vascular function

AlxHR75, an indirect marker of arterial stiffness, was significantly improved after ten days of cold acclimation measured in the overnight fasted state (pre T0 22.57 ± 1.36 % vs. post T0 19.84 ± 1.96 %, $p=0.03$, **Figure 4A**). Before cold acclimation, AlxHR75 tended to decrease upon the first meal ingestion (T120) and this meal-induced effect became significant after the second meal (T300). No statistically significant meal-effects on AlxHR75 were observed after cold acclimation (pre T120 15.63 ± 2.80 % vs. post T120 18.26 ± 1.67 % $p=0.50$ and pre T300 14.94 ± 2.55 % vs. post T300 16.33 ± 2.70 % $p=0.82$, **Figure 4A**). Postprandial changes in AlxHR75 were also not significant different before and after cold acclimation (pre delta 120 -6.94 ± 2.31 vs. post delta 120 -1.58 ± 1.96 , $p=0.16$, pre delta 300 -7.63 ± 1.83 vs. post delta 300 -3.51 ± 1.91 , $p=0.30$, **Figure 4B**).

Figure 4. Vascular function markers

A: Aortic augmentation index (AlxHR75) at timepoints T0, T120, T300. **B:** AlxHR75 delta's T0-120 and T0-300 expressed as percentage points. **C:** Pulse wave velocity (PWV_{c-f}) at timepoints T0, T120, T300 (n=8 at T300). **D:** PWV_{c-f} delta's T0-120 and T0-300 (n=8 at delta T0-300). **E:** Arteriolar-to-venular ratio of the retinal vessels in the right eye (n=8). Black bars represent data from the meal test before cold acclimation, grey bars represent data from the meal test after cold acclimation. Data shown are shown as mean $\pm$ SE. * $p<0.05$, # $p<0.10$.

The current non-invasive gold standard technique to measure arterial stiffness (PWV_{c-f}), was however not affected by cold acclimation in the overnight fasted state PWV_{c-f} (pre T0 12.36 ± 0.61 vs. post T0 11.99 ± 0.57 , $p=0.36$, **Figure 4C**). As expected, no meal-induced effects were observed (see **Figure 4D**). Finally, ten days of cold acclimation did not significantly affect fasting retinal vessel diameter, as the arteriolar width (pre $120.83 \pm 7.48 \mu m$ vs. post $120.52 \pm 7.89 \mu m$, $p=0.95$), venular width (pre $209.19 \pm 10.73 \mu m$ vs. post $207.17 \pm 10.07 \mu m$, $p=0.23$) and the arteriolar-to-venular ratio (pre 0.58 ± 0.02 vs. post 0.58 ± 0.02 , $p=0.84$, **Figure 4E**) did not change.

Intrahepatic lipid content

As we hypothesized that cold acclimation could affect postprandial lipid metabolism, we also investigated if cold acclimation affects intrahepatic lipid content. However, intrahepatic lipid content was not different before and directly after cold acclimation (pre 6.1 ± 4.3 vs. post 7.0 ± 4.0 %, $p=0.22$, $n=8$, **Figure 5D**).

Figure 5. Insulin sensitivity and intrahepatic lipid content

A: Whole body insulin sensitivity. **B:** Suppression of hepatic endogenous glucose production. **C:** HbA1c before the cold acclimation and 2 months after the cold acclimation. **D:** Intrahepatic lipid content. Black bars represent before cold acclimation, grey bars represent directly after cold acclimation, white bars represent ten days after the cold acclimation, dashed bar represents two months after the cold acclimation. Data are shown as mean $\pm$ SE.

Insulin sensitivity

We previously demonstrated that cold acclimation improved insulin sensitivity when measured directly after ten days of cold acclimation (13). Here, we aimed to investigate if this increase in insulin sensitivity would be sustained for ten days after cessation of the cold acclimation intervention. Interestingly, plasma insulin levels during the high insulin phase of the clamp were significantly different between test days ($p=0.02$) with significantly higher values directly after the cold acclimation compared to before cold acclimation ($p=0.02$, **Supplementary Table 3**), and therefore we calculated Si as a measure of insulin sensitivity. However, whole body insulin stimulated glucose uptake (Rd glucose high insulin minus baseline) corrected for plasma insulin levels during the clamp (Si), was not different before and directly after cold acclimation nor on the longterm ($p=0.53$, **Figure 5A**). Hepatic insulin sensitivity during the low insulin phase was also not affected by cold acclimation ($p=0.28$, **Figure 5B**), however during the high insulin phase, EGP suppression was significantly lower directly after cold acclimation compared to before and on the longterm (pre 88.06 ± 7.00 % vs. post 77.60 ± 7.45 % vs. longterm 88.39 ± 3.44 %, $p=0.04$). HbA1c, a marker of long-term glucose homeostasis, was not different before cold acclimation compared to two months after cold acclimation (pre 7.3 ± 0.3 % vs. two months later 7.2 ± 0.3 %, $p=0.71$, **Figure 5C**).

Skeletal muscle GLUT4 translocation

The lack of effect of cold induced non-shivering thermogenesis on insulin sensitivity contrasts our previous study, in which we found that the increased insulin sensitivity was due to enhanced GLUT4 translocation in skeletal muscle (13). Consistent with a lack of effect of cold induced NST on insulin sensitivity, GLUT4 intensity at the membrane measured in muscle biopsies taken in the non-insulin stimulated condition, was also not affected by cold induced NST (pre 27.37 ± 3.05 vs. post 27.63 ± 2.28 vs. longterm 27.61 ± 3.30 A.U., $p=0.77$, $n=7$, **Figure 6B**). Cytosolic GLUT4 intensity was not changed either (pre 20.21 ± 1.89 vs. post 20.71 ± 1.33 vs. long term 20.46 ± 1.90 A.U., $p=0.96$).

Skeletal muscle shivering markers

Because we could not replicate our previously reported positive effects of cold acclimation on insulin sensitivity and GLUT4 translocation (13), we decided to investigate if (the lack of) shivering may have contributed to the discrepancy in glucose homeostasis results between both studies. As a first indication, we see a distinct lack of self-reported shivering in the current study as compared to the previous one (13), which is visualized in **Figure 6A**. Next, we measured mRNA expression of eight genes that we recently identified (unpublished data) to be

associated with muscle activation and -remodeling by comparing gene expression patterns – using micro array analysis – between our previous cold acclimation study (13) and an exercise training study (14). As can be seen in **Figure 6C**, the change in mRNA expression of alpha actin cardiac muscle (ACTC1), cholinergic receptor nicotinic delta subunit (CHRND), coiled-coil domain containing 80 (CCDC80) and cholinergic receptor nicotinic alpha 1 subunit (CHRNA1), upon ten days of cold acclimation different between the two cold-acclimation studies, with basically no change in these genes in the current study (ACTC1 -0.2 ± 0.1 vs. 1.3 ± 1.8 , $p=0.02$; CHRND -0.0 ± 0.1 vs. 3.4 ± 2.5 , $p=0.02$; CCDC80 0.1 ± 0.1 vs. 3.8 ± 3.1 $p=0.02$; CHRNA1 -0.1 ± 0.1 vs. 2.2 ± 1.9 arb. unit, $p=0.02$, in the current and the Hanssen et al. study (13) respectively, **Figure 6C**). Although similar differences were observed for RRAD, MYH8, LYRM3 and CCDC39, these differences did not reach statistical significance ($p=0.12$, $p=0.17$, $p=0.35$ and $p=0.09$ respectively, **Figure 6C**).

Figure 6. Skeletal muscle shivering markers

A: Self-reported shivering measured with VAS scales at selected timepoints (from T=0 minutes until T=360 minutes) during day 3 and day 10 of the cold acclimation period, shown as AUC. The dashed line indicates the self-reported shivering AUC from Hanssen et al. (13) at day 3 **B:** GLUT4 intensity at the skeletal muscle membrane, expressed as arbitrary units (A.U.). **C:** Delta gene expression shivering markers, measured in muscle samples from the current study in black (n=9) and from Hanssen et al. (13) in blue (n=7) before and directly after cold acclimation. Data are shown as mean $\pm$ SE. * $p<0.05$, # $p<0.10$.

DISCUSSION

Previous studies investigating cold acclimation in humans have shown a potential to treat obesity and T2DM via an increase in energy expenditure (4-7, 12, 13, 36-38) and insulin sensitivity (13), acting through NST in brown adipose tissue and skeletal muscle. Therefore, we hypothesized that cold acclimation with NST could also be beneficial for postprandial metabolism and reduce cardiovascular risk. To this end, we primarily investigated the effects of ten-day cold acclimation without shivering on cardiovascular risk markers, including postprandial glucose and lipid metabolism and markers for arterial stiffness. Moreover, we investigated the long-term effects of cold acclimation without shivering on insulin sensitivity.

Acute cold exposure with and without shivering has frequently been shown to increase energy expenditure, reflected by an increase in basal metabolic rate (4, 5, 10, 11, 37-42). Yet, consistent with other previous reports (4, 6, 7, 12, 13, 36, 38, 43), we did not observe an increase in basal metabolic rate, measured under thermoneutral conditions after cold acclimation with NST. However, our results show a change in fasting substrate selection after cold acclimation with NST, with a decrease in fat oxidation and an increase in carbohydrate oxidation. Furthermore, as also reported before (12), postprandial energy expenditure, which reflects diet induced thermogenesis, was higher in the meal test after cold acclimation compared than in the meal test prior to cold acclimation. Other evidence suggests that acute cold exposure (5, 11, 16, 39, 41), but not cold acclimation (5), increases glucose oxidation, fat oxidation and lipid clearance. In agreement with previous work (5, 42), we did not observe changes in fasting plasma lipid levels, postprandial substrate oxidation or postprandial triglyceride response. We only observed a small, albeit significant decrease in plasma fatty acid levels during the first step of the meal test after cold acclimation. Furthermore, no effects of cold acclimation with NST were observed on fasted plasma glucose and insulin levels, consistent with findings after acute cold exposure (42). These results indicate that cold acclimation under non-shivering conditions only had marginal effects on postprandial glucose and lipid metabolism.

Furthermore, we observed a significant improvement in the augmentation index, indicating an indirect reduction in arterial stiffness. However, we did not see any change in PWV_{c-f}. This indicates that the effects of ten days of cold acclimation with NST might only affect peripheral resistance arteries and not stiffness of the large elastic arteries, such as the aorta. It is likely that the study period of ten days was too short to induce pronounced effects on arterial vessel wall properties. Alternatively,

cold exposure can increase blood pressure – a main determinant of the PWV_{c-f} – through vasoconstriction (44), and an increased blood pressure during cold exposure could mitigate potential beneficial effects that could be expected from the cold acclimation.

We previously showed marked effects of cold acclimation on insulin sensitivity in T2DM patients (13). Here we aimed to investigate if these effects would be retained for a longer period of time after the last cold exposure under non-shivering conditions. Thus, we performed a hyperinsulinemic euglycemic clamp before cold acclimation, directly (one day) after cold acclimation and ten days after the last cold exposure. Surprisingly, we could not replicate the increase in insulin sensitivity directly following the ten-day cold acclimation period, and also no effects on the long term were observed. Since the increase in insulin sensitivity in our previous study (13) was very marked (40% increase), observed in all participants, and was accompanied by marked increases in muscle GLUT4 translocation, we carefully evaluated the differences between the two studies. Thus, in both studies patients with T2DM were investigated before and after ten days of cold exposure, following a similar design. One difference in the current study design was the inclusion of a meal test as the primary outcome parameter. This meal test was performed on the day following the ten-day cold exposure. Consequently, the clamp in the current study was performed three days after the meal test, with two days of additional cold exposure in between. We can hence not exclude a carry-over effect of the meal test on the results of the clamp. It should be noted though, that postprandial glucose metabolism measured during the meal tests, which can be seen as a marker of insulin sensitivity, was also not affected by cold acclimation.

Another difference between the two studies was that in the current study we specifically aimed to investigate the effect of NST and aimed to prevent shivering. To this end, the room temperature during cold exposure was approximately 1.4 °C higher compared to our previous study (13). Furthermore, participants were provided with extra clothing when shivering occurred to prevent shivering thermogenesis. While this strategy was more effective in preventing shivering and/or tense muscle compared to our previous study (13), as indicated by the self-reported shivering questionnaires taken during the cold acclimation, it may also have affected the clamp outcome. Furthermore, we previously reported a marked increase in GLUT4 translocation in the overnight fasted state (so in the absence of elevated insulin levels) and under thermoneutral conditions after cold acclimation (7, 13). Classically, this increase in GLUT4 in the cell membrane in the absence of insulin stimulation is attributed to muscle contraction, which is required for shivering or to increase muscle tension (45).

Consistent with the self-reported absence of shivering, no effect of cold acclimation on GLUT4 translocation was found in the current study. To further investigate the possible effects of shivering that we may have missed in our previous study (13), we performed gene-expression analyses in skeletal muscle biopsies obtained before and directly after the cold acclimation in both studies. Results of these analyses show upregulation of muscle-contraction-related-genes in our previous study (13) but no change in the current study. Those genes were selected from micro-array data obtained after cold exposure (13) and compared with an exercise training study (14). This comparison revealed overlap in skeletal muscle gene expression related to muscle contraction pathways (unpublished data). Interestingly, all previous published studies that observed improvements in insulin sensitivity after cold acclimation, included at least a few days of (mild) shivering before NST occurred (4, 5, 7, 13, 38). Taken together, the findings reported here and in literature (4, 10, 46), suggest that some mild form of cold-induced shivering is needed to trigger beneficial effects on skeletal muscle insulin sensitivity.

In conclusion, we here show that a ten-day cold acclimation period at 16-17 °C with NST does not induce metabolic improvements nor reduce cardiovascular risk markers in obese men and women with T2DM. The lack of effects in the current study are probably due to the absence of shivering compared to previous studies. Therefore, further research could focus on the potential of (mild) shivering thermogenesis on metabolic health, as ten days of cold acclimation without shivering at 16-17 °C in obese men and women with T2DM is insufficient to induce positive effects on human metabolism.

SUPPLEMENTARY MATERIAL FOR CHAPTER 4

Supplementary Table 1. Participant characteristics regarding diabetes

Participant	Diabetes duration (years)	Diabetes medication
1	15	Metformin 850mg, 3x/day Gliclazide 80mg, 2x/day
2	14	Metformin 500mg, 2x/day
3	15	Metformin 1000mg, 2x/day Gliclazide 30mg, 2x/day
4	6	Metformin 1000mg, 3x/day
5	8	Metformin 850mg 1x/day Glimepiride 2mg, 1x/day Liraglutide 6mg, 18eh, 1x/day
6	5	Metformin 850mg, 2x/day
7	9	Metformin 500mg, 2x/day
8	7	Metformin 500mg, 3x/day Gliclazide 30mg, 1x/day
9	1	Gliclazide 80mg, 1x/day

Supplementary Table 2. Nutritional information of meal test shake

	Content	Energy %
Energy	755 kcal	100
Fat	50.8 gr	60.6
Saturated fat	26.1 gr	31.1
Unsaturated fat	23.8 gr	28.4
Cholesterol	0.9 gr	1.1
Carbohydrate	62.3 gr	33.0
Protein	10.9 gr	5.8

Supplementary Table 3. Plasma levels and substrate kinetics measured during the clamps

Parameter	Clamp pre	Clamp post	Clamp longterm	p-value
Plasma Glucose (mmol/l)				
Baseline	7.77 ± 0.54	7.98 ± 0.45	7.91 ± 0.54	0.99
Low insulin	5.99 ± 0.25	5.78 ± 0.31	5.63 ± 0.17	0.21
High insulin	5.21 ± 0.12	5.11 ± 0.11	5.04 ± 0.08	0.69
Plasma Insulin (mU/l)				
Baseline	6.32 ± 1.992	6.99 ± 1.48	6.56 ± 1.71	0.69
Low insulin	14.09 ± 1.87	12.64 ± 1.56	12.62 ± 0.88	0.57
High insulin	62.50 ± 3.24 ^C	59.34 ± 4.13	56.03 ± 2.68 ^C	0.02*
Energy expenditure (kJ/min)				
Baseline	4.82 ± 0.25	4.91 ± 0.27	4.86 ± 0.20	0.79
Low insulin	4.86 ± 0.34	4.92 ± 0.28	4.75 ± 0.24	0.24
High insulin	4.67 ± 0.30 ^B	4.89 ± 0.27 ^{B,D}	4.67 ± 0.18 ^D	0.02*
Carbohydrate oxidation (μmol/kg/min)				
Baseline	3.76 ± 0.47 ^A	4.97 ± 0.68 ^A	4.68 ± 0.68	<0.01**
Low insulin	7.27 ± 0.79	7.83 ± 0.84	7.15 ± 0.77	0.79
High insulin	10.36 ± 1.15	11.07 ± 1.21	9.73 ± 1.41	0.30
Fatty acid oxidation (μmol/kg/min)				
Baseline	3.80 ± 0.17 ^A	3.57 ± 0.17 ^{A,D}	3.71 ± 0.19 ^D	<0.01**
Low insulin	2.81 ± 0.23	2.86 ± 0.24	2.96 ± 0.16	0.87
High insulin	2.14 ± 0.24	2.16 ± 0.21	2.39 ± 0.26	0.99

Abbreviations: Ra, rate of appearance; Rd, rate of disappearance; S_i, whole body insulin sensitivity; EGP, endogenous glucose production; NOGD, non-oxidative glucose disposal; FFA, free fatty acids. Data are expressed as mean ± SE. **p<0.01, *p<0.05, ^Asignificant difference (p<0.01) between Clamp pre and Clamp post, ^Bsignificant difference (p<0.05) between Clamp pre and Clamp post, ^Csignificant difference (p<0.05) between Clamp pre and Clamp post, ^Dtrend in difference (p<0.10) between Clamp post and Clamp longterm.

Supplementary Table 4. Plasma levels and substrate kinetics measured during the meal test

Parameter	Meal test pre	Meal test post	p-value
Plasma Glucose			
AUC	4433 ± 236	4285 ± 170	0.43
iAUC	860 ± 171	747 ± 159	0.25
Plasma Insulin			
AUC	15826 ± 1550	15640 ± 1458	0.65
iAUC	9457 ± 1097	9060 ± 1119	0.36
Plasma Triglycerides			
AUC	1339 ± 109	1411 ± 123	0.50
iAUC	655 ± 89	664 ± 67	0.36
Plasma Free fatty acids			
AUC	214852 ± 9104	202701 ± 10011	0.16
iAUC	13182 ± 6077	23031 ± 7650	0.20
AUC first meal	94228 ± 5115	86014 ± 4545	0.04*
AUC second meal	120624 ± 5448	106486 ± 11976	0.30
AUC Energy expenditure			
AUC	2620 ± 145	2752 ± 168	0.03*
iAUC	316 ± 37	355 ± 22	0.69
AUC Carbohydrate oxidation			
AUC	3328 ± 371	3866 ± 550	0.44
iAUC	726 ± 152	998 ± 103	0.22
AUC Fatty acid oxidation			
AUC	1688 ± 92	1667 ± 78	>0.99
iAUC	611 ± 159	526 ± 63	0.94

Abbreviations: AUC, area under the curve; iAUC, incremental area under the curve.

Data are expressed as mean ± SE. *p<0.05.

Supplementary Figure 1. Subjective responses to cold acclimation

Self-reported thermal sensation (**A**) and thermal comfort (**B**) measured with VAS scales at selected timepoints (from $t=0$ min until $t=360$ min) during day 3 and day 10 of the cold acclimation period, shown as AUC. Data are expressed as mean $\pm$ SE.

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Chapter 4

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CHAPTER 5

Replacing sedentary time with standing and walking is similar effective in enhancing insulin sensitivity as moderate-to-vigorous exercise

Carlijn M.E. Remie, Lena Bilet, Bernard M.F.M. Duvivier, Maaike Bergman, Kay H.M. Roumans, Stephanie M.D. Huysmans, Evelyn Zacharewicz, Vera H.W. de Wit, Niels J. Connell, Bas Havekes, Johanna A. Jörgensen, Kim Brouwers, Hans H.C.M. Savelberg Vera B. Schrauwen-Hinderling, Joris Hoeks, Matthijs K.C. Hesselink, Esther Phielix, Patrick Schrauwen

In preparation

ABSTRACT

Background & Aims: Sedentary behavior has been associated with an increased risk to develop type 2 diabetes, metabolic syndrome and cardiovascular diseases. Replacing sitting time by activities such as standing and walking or by physical exercise have both been proven effective in improving aspects of metabolic health, including glucose homeostasis. If these two physical activity regimes act via similar mechanisms remains however to be investigated.

Methods: A randomized, cross-over intervention study was conducted in twelve overweight/obese women. Participants performed three activity regimes of each four days: 1) sit regime (sitting 14 h/day), 2) exercise regime (sitting 13 h/day, exercise 1 h/day) and 3) sit less regime (sitting 9 h/day, standing 4 h/day and walking 3 h/day). After each regime insulin sensitivity was determined using a hyperinsulinemic-euglycemic clamp combined with muscle biopsies, intrahepatic lipid content was determined by MRS and substrate oxidation by indirect calorimetry. *Ex vivo* mitochondrial function in muscle biopsies was measured using high-resolution respirometry.

Results: Peripheral insulin sensitivity was significantly higher after the exercise regime compared to the sit regime (ΔR_d 28.2 ± 3.3 $\mu\text{mol/kg/min}$ vs. 20.7 ± 3.7 $\mu\text{mol/kg/min}$, $p < 0.01$) and tended to be higher in the sit less regime (ΔR_d 25.5 ± 2.8 $\mu\text{mol/kg/min}$, $p = 0.08$). This increase in peripheral insulin sensitivity was mainly due to a higher NOGD after the exercise and sit less regimes compared to the sit regime, with no change in oxidative glucose disposal. Muscle mitochondrial respiratory capacity was unaffected by the three activity regimes. Hepatic insulin sensitivity neither intrahepatic lipid content was different between the three activity regimes.

Conclusion: Replacing sitting time by standing and light walking has comparable positive effects on peripheral insulin sensitivity compared to vigorous exercise, which could not be explained by improvements in mitochondrial function.

INTRODUCTION

Sedentary behaviour has frequently been reported to be a risk factor for type 2 diabetes, metabolic syndrome, cardiovascular disease and all-cause mortality (1-3). As such, physical activity or exercise has been an important cornerstone in the treatment of type 2 diabetes. Indeed, a wide variety of exercise interventions – including endurance training, resistance training and/or high intensity interval training, or any combination – have been shown to be effective in improving insulin sensitivity and glycemic control (4). Although this treatment strategy has been proven to be very effective, the compliance to maintain an active lifestyle with regular moderate-to-vigorous physical activity has been shown to be low in the general European and US adult population (5, 6), with even lower compliance in people diagnosed with type 2 diabetes or adults at risk to develop this disease (7).

Alternative, more assessable approaches are therefore needed for the treatment and prevention of type 2 diabetes. Strategies to reduce sedentary behaviour involving light physical activities are generally considered less demanding than structured exercise and hence are more likely to have long-term compliance (8). Indeed, numerous human interventions studies in which daily activities were manipulated, have shown promising effects of reducing sedentary time when replaced with standing (9, 10), walking (9-13) or short exercise bouts (13) on postprandial plasma glucose and insulin responses. We recently showed improvements in glycemic control already after a 4-day intervention in which two sitting replacement strategies were compared to a sedentary lifestyle (14-16). Replacing sitting time with several hours of standing and walking was effective in improving glucose homeostasis – as determined by an OGTT – in healthy young adults, healthy obese men and women and patients with type 2 diabetes, when compared to sedentary behaviour (14-16). Moreover, in these studies, replacing one hour of sitting time with one hour of exercise resulted in a near significant reduction of 24h glucose iAUC, a reduction that was even more pronounced when sitting time was replaced by several hours of standing and walking (16). At this stage, these promising results for light physical activities provide putative novel tools for improvements in metabolic control of type 2 diabetes.

So far, the exact mechanisms underlying the beneficial effects of breaking sedentary time are unknown. Mechanisms underlying the exercise-induced glucose lowering effect have been well established; it has been shown that next to insulin, muscle contraction is also able to stimulate GLUT4 translocation, thereby facilitating glucose uptake in skeletal muscle (17, 18). In addition, it has been shown that exercise training is an effective tool to enhance skeletal muscle mitochondrial function, which

coincides with better peripheral insulin sensitivity (19). Whether the observed improvements in glucose homeostasis by replacing sedentary time with standing and walking are exerted via similar pathways has not been extensively studied. Analysis in human muscle biopsies, obtained after interrupting prolonged sitting, revealed changes in skeletal muscle gene expression comparable to exercise induced changes (20, 21), but no functional measurements were performed. Furthermore, habitual physical activity in daily life has been correlated positively with markers of mitochondrial capacity (22), which might suggest a role for mitochondria in the observed improvement in insulin sensitivity upon sitting less, but data directly testing this hypothesis is lacking

Therefore, in the present study, the primary aim was to investigate whether an improved glucose homeostasis upon the replacement of sedentary time by standing/walking – as observed by Duvivier et al. (14-16) – can be explained by enhanced peripheral insulin sensitivity and skeletal muscle mitochondrial function as potential underlying factors. Furthermore, we investigated whether hepatic insulin sensitivity and hepatic lipid content are affected by replacing sedentary time by standing/walking. These outcomes were also compared to replacing sedentary time by a single exercise bout per day.

METHODS

Participants

Recruitment and data collection took place between December 2017 and August 2019 in the vicinity of Maastricht. In total 26 female volunteers were screened, of who thirteen were found eligible and included in the study. Of these, twelve participants completed the study as one participant dropped out because of personal reasons. The screening included an assessment of blood biochemistry, electrocardiography, anthropometry measurements and a questionnaire including Baecke physical activity questionnaire (23). Inclusion criteria were healthy postmenopausal women, 45-70 years of age, BMI 25-35 kg/m², sedentary lifestyle (<3 h exercise per week), non-smoking for at least 6 months, alcohol use of ≤ 2 servings per day, stable body weight for at least 6 months.

Study design

A randomized three-arm cross-over intervention study was performed, as previously published elsewhere (**Figure 1**) (16). In short, participants underwent all three study arms including four-day activity regimes: 1) sit (SIT), 2) exercise (EXE), 3) sit less (SL).

Prior to the three activity regimes baseline measurements were performed for participant characterization. During the sit regime, participants were instructed to sit 14 h/day, stand 1 h/day, walk 1 h/day and to spend 8 h/day sleeping or lying. During the exercise regime 1 h/day of sitting was replaced by 1 h/day of vigorous supervised exercise. The rest of the days in the exercise regime were spend similarly as during the sit regime. During the sit less regime 5 h/day of sitting was replaced by 3 h of standing and 2 h of walking. Participants were advised to spread the standing and walking hours over the day. The exercise and sit less regime differed largely in time spent sitting but were designed to have equal total daily energy expenditure. The sit and exercise regime differed only 1 h in sitting time but had substantially different total daily energy expenditure. Any other physical activities besides sitting, standing, walking and the supervised exercise session were limited as much as possible. Directly after the four-day activity regimes, on day five, several measurements were performed. A washout period of at least nine days and maximum 23 days was applied between the activity regimes.

Figure 1. Study design

A randomized three-arm cross-over design, in which participants followed three activity regimes in a random order: sit regime, exercise regime and sit less regime, with a washout period of 9-23 days between arms. Each activity regime lasted four days and measurements were performed on day 5. Time per day (expressed in hours) spend walking, standing, sitting, exercising and sleeping are shown in the figure.

Standardization of dietary intake

Dietary intake was kept similar during all three regimes. Participants were instructed to adhere to their normal dietary habits. The consumption of alcohol and caffeine rich drinks were not allowed during the five intervention days. During the first activity regime, participants carefully recorded both time and content of all consumptions in a diary. The researcher returned these dietary records to the participants who were instructed to consume exactly the same diet in the second and third activity regime. In order to standardize the last twelve hours before this test day, participants were instructed to consume a standardized dinner on the evening of day four before 20:00 h and refrain from food and drinks afterwards except for water.

Physical activity assessment

Physical activity and body posture allocation was measured 24 h/day using an activPAL activity monitor (PAL technologies, Glasgow, Scotland). The activPAL was waterproofed packed and attached to the anterior thigh of the participant. Before the start of the actual activity regimes, participants first wore the activPAL monitor for five consecutive days, including three week and two weekend days, to assess habitual physical activity level. The activPAL discriminates between time being inactive (sitting or lying) or being active (standing and walking) as well as recorded step number and cadence. In addition, participants recorded their physical activity in a diary and these data were compared with activPAL readouts to formulate tailor-made instructions on how to alter daily activities to those prescribed for the three different activity regimes. This was done in order to guarantee optimal compliance with each activity regime. Sleeping time was determined based on diary data. Daily energy expenditure was estimated using the 24 h activPAL data expressed in metabolic equivalents (METs) and with separate calculations for the exercise session as described earlier (16).

Participant characteristics

In order to assess participant characteristics, body mass and body volume were assessed using air-displacement plethysmography using the BodPod device (Cosmed, Italy, Rome) according to the manufacturer's instructions (24), from which body composition (fat mass and fat free mass) was calculated. Waist and hip circumference were measured and the average of 3 values were reported. Next, *in vivo* maximal oxygen consumption (VO_2max) and maximal power (Wattmax) were assessed during an incremental exercise test on an ergometer (25). The highest average oxygen consumption over 25 seconds was used as the VO_2max , and the corresponding adjusted wattage as Wattmax. The VO_2max reflects the participant's physical fitness and 60% of the Wattmax was applied during the supervised exercise session. All the measurements were performed in the morning after an overnight fast

of at least 10 hours with a minimum of three days before the start of the activity regimes.

Intrahepatic lipid quantification by MR spectroscopy

At 07:00 h in the morning of the test day after ending the activity regime period, proton magnetic resonance spectroscopy (¹H-MRS) was used to quantify intrahepatic lipid content (IHL). All participants were relocated in a wheelchair from the research facility to and from the radiology department. Measurements were performed on a 3.0T whole body scanner (Achieva Tx, Philips Healthcare, Best, The Netherlands). Spectra were acquired as described before (26). Values are given as T2 corrected ratios of the CH₂ peak relative to the unsuppressed water peak, expressed as percentage. One participant failed to undergo this measurement, due to claustrophobia and another participant was excluded due to a technical error.

Skeletal muscle biopsies

Following the MRS measurement, a muscle biopsy was taken (08:30 h) from the m. vastus lateralis under local anesthesia (1% lidocaine, without epinephrine) using the Bergström technique (27). Muscle tissue was used for mitochondrial respiratory measurements.

Skeletal muscle mitochondrial respiration

Muscle tissue obtained with the biopsy was immediately placed in an ice-cold preservation medium (BIOPS, OROBOROS Instruments, Innsbruck, Austria). Muscle fibers were permeabilized using saponin. After permeabilization and several washing steps with an ice-cold mitochondrial respiration buffer (MiR05, OROBOROS Instruments, Innsbruck, Austria), muscle fibers were transferred into the high-resolution respirometer (Oxygraph, OROBOROS Instruments, Innsbruck, Austria). Thereafter *ex vivo* mitochondrial respiration was determined by measuring oxygen consumption rate (pmol/mg wet weight per second) upon addition of several substrates using high-resolution respirometry as previously described (28). All measurements were performed in quadruplicate and traces with a cytochrome C response above 15% were excluded, indicating disrupted integrity of the inner mitochondrial membrane. Respirometry could not be determined in one participant due to difficulties with the muscle biopsy.

Plasma triglycerides

Triglycerides (Sigma, Zwijndrecht, The Netherlands) were analyzed using a Pentra 400 in EDTA plasma from fasting blood samples taken in the morning between 06:30 and 07:00 h.

Blood pressure

Resting systolic (SBP) and diastolic (DBP) blood pressure as well as heart rate (HR) were measured in supine position the morning, every 5 minutes between 09:00 and 09:30 h, at least 10 minutes after taking the muscle biopsy and before the start of the clamp, using a semi-continuous blood pressure monitoring device (OmronX, CEMEX Medische Techniek, Nieuwegein, The Netherlands). The average of the seven measurements were reported.

Hyperinsulinemic euglycemic clamp

After the ^1H -MRS, muscle biopsy and blood pressure measurement, a two-step hyperinsulinemic euglycemic clamp was performed to determine hepatic and peripheral insulin sensitivity (29). During the ^1H -MRS measurement (at 07:00 h) a primed, continuous D-[6,6- $^2\text{H}_2$] glucose tracer infusion was started (0.04 mg/kg/min). Upon 3 h of D2-glucose pre-infusion, a 3 h primed low dose continuous insulin infusion was started (10 mU/m² per min) to assess hepatic insulin sensitivity. Subsequently, a 2.5 h primed high dose continuous insulin infusion was started (40 mU/m² per min) to measure peripheral insulin sensitivity. Blood was frequently sampled from arterialized blood to monitor glucose levels in order to maintain euglycemia (~5.0 - 5.5 mmol/l). During the last 30 minutes of the baseline, low and high insulin phases, steady state was reached and blood samples were collected for determination of glucose tracer kinetics. During these phases also whole body substrate utilization was measured using indirect calorimetry (Omnical, Maastricht Instruments, Maastricht, The Netherlands) (30). Due to technical reasons, one participant was excluded from the low insulin phase clamp analysis and another participant was excluded from the high insulin phase clamp analysis.

Calculations

During the clamp, energy expenditure, carbohydrate (CHO) oxidation and fatty acid (FA) oxidation rates were calculated using equations based on the measured oxygen and carbon dioxide concentrations with the assumption that protein oxidation was negligible (31, 32). The respiratory exchange ratio (RER) was calculated by the carbon dioxide/oxygen ratio. Steele's single pool non-steady state equations were used, to correct for small differences in glucose concentrations, to calculate glucose appearance (Ra) and glucose disposal (Rd) (33). Volume of distribution was assumed to be 0.160 L/kg for glucose. Insulin-stimulated glucose disposal (ΔRd) was calculated by the difference between Rd under insulin-stimulated conditions minus the Rd under basal non-insulin-stimulated conditions. Endogenous glucose production (EGP) was calculated as Ra minus exogenous glucose infusion rate. The percentage insulin-suppressed EGP was calculated as the percentage insulin-

suppressed EGP from the basal EGP. Non-oxidative glucose disposal (NOGD) was calculated as Rd minus carbohydrate oxidation. The change in insulin stimulated NOGD (Δ NOGD) was calculated as the NOGD during insulin minus the basal measured NOGD.

Statistical analyses

Participant characteristics are reported as mean $\pm$ SD, other outcome measures are reported as mean $\pm$ SE. Data are presented for n=12, unless otherwise indicated. All data were evaluated for normal distribution. Differences between interventions were analyzed with a Repeated Measures ANOVA for parametric data and with a Friedman test for non-parametric data. Post hoc Bonferroni and Dunn's correction, respectively, was applied to correct for multiple testing. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using IBM SPSS version 23.0 for MacOS.

Ethical approval

The study was conducted in accordance with the principles of the declaration of Helsinki and approved by the Ethics Committee of the Maastricht University Medical Center. The study was registered at <https://clinicaltrials.gov> (NCT03912922). All participants provided written informed consent before screening.

RESULTS

Participant population and study compliance

Twelve healthy overweight and obese women (age 64 ± 5 years; BMI 29.2 ± 2.9 kg/m²) participated and completed the study. Participants were non-smokers, had no active diseases, and had a sedentary lifestyle according to the Baecke questionnaire (6.55 ± 0.95) (**Table 1**). Medication use of the participants did not interfere with the main outcomes of this study (**Supplementary Table 1**). Habitual daily physical activity level showed an average habitual sedentary time (including sleeping time) of 17.57 ± 1.15 h, standing time 4.33 ± 1.23 h and walking time 2.10 ± 0.62 h, with an average of 10141 ± 3250 steps per day (**Table 1**).

The three activity regimes were successfully implemented, as the time spent sitting, standing, walking and exercising were in accordance with the study design (**Table 2**). The time spent standing (4.0 ± 0.1 h) and walking (3.0 ± 0.1 h) were significantly higher in the SL regime compared to the SIT ($p < 0.01$) and EXE regimes ($p < 0.01$),

Table 1. Participant characteristics

Parameter	Mean $\pm$ SD
Age (years)	64 $\pm$ 5
Body weight (kg)	80.1 $\pm$ 9.2
BMI (kg/m ²)	29.2 $\pm$ 2.9
Fat mass (%)	46.1 $\pm$ 11.3
Waist circumference (cm)	97 $\pm$ 9
Hip circumference (cm)	110 $\pm$ 8
Fasting glucose (mmol/l)	5.4 $\pm$ 0.5
Systolic blood pressure	133 $\pm$ 16
Diastolic blood pressure	85 $\pm$ 9
VO ₂ max (ml/min/kg)	23.8 $\pm$ 4.3
Physical activity level (Baecke score)	6.55 $\pm$ 0.95
Habitual sitting + sleeping time (h)	17.6 $\pm$ 1.2
Habitual standing time (h)	4.3 $\pm$ 1.2
Habitual walking time (h)	2.1 $\pm$ 0.6
Habitual steps/day (n)	10141 $\pm$ 3250
Habitual cadence (steps/min)	80 $\pm$ 5

Abbreviations: BMI, body mass index; VO₂max, maximal oxygen consumption. N=12.

Table 2). The times spent standing (SIT 1.3 $\pm$ 0.1 h, EXE 1.2 $\pm$ 0.1 h) and walking (SIT 1.0 $\pm$ 0.0 h, EXE 1.0 $\pm$ 0.0 h) were not significantly different between the SIT and EXE regimes ($p=0.99$, **Table 2**). During the EXE regime, on average 1.0 $\pm$ 0.1 hours of sitting per day was substituted by cycling during a supervised exercise session at an intensity of on average 82 $\pm$ 5 Watt, corresponding to on average 4.60 $\pm$ 0.15 MET. The number of steps per day were significantly higher in the SL regime (16875 $\pm$ 463 steps/day) compared to the SIT (4878 $\pm$ 240 steps/day, $p<0.01$) and EXE regime (5082 $\pm$ 165 steps/day, $p<0.01$, **Table 2**). Furthermore, the walking cadence was significantly higher in the SL regime (94 $\pm$ 2 steps/min) compared to the SIT (85 $\pm$ 3 steps/min, $p<0.01$) and EXE (86 $\pm$ 2 steps/min, $p<0.01$) regimes (**Table 2**). The estimated energy expenditure was lower in the SIT regime (32.0 $\pm$ 0.1 MET/day) compared to the EXE (36.6 $\pm$ 0.2 MET/day, $p<0.0001$) and SL regimes (37.0 $\pm$ 0.2 MET/day, $p<0.01$, **Table 2**). The amount of time spent sleeping was not different between the three regimes with an average of 8.0 $\pm$ 0.1 hours per day ($p=0.28$, **Table 2**). The self-reported dietary intake was not different between the three activity regimes (data not shown).

Table 2. Physical activity during activity regimes

Parameter	SIT	EXE	SL	p-value	p-value SIT vs EXE	p-value SIT vs SL	p-value EXE vs SL
Sitting (h/day)	13.6 ± 0.2	12.5 ± 0.3	9.2 ± 0.2	<0.01*	<0.01*	<0.01*	<0.01*
Standing (h/day)	1.3 ± 0.1	1.2 ± 0.1	4.0 ± 0.1	<0.01*	0.99	<0.01*	<0.01*
Walking (h/day)	1.0 ± 0.0	1.0 ± 0.0	3.0 ± 0.1	<0.01*	0.46	<0.01*	<0.01*
Exercise (h/day)	-	1.0 ± 0.1	-	-	-	-	-
Sleeping (h/day)	8.1 ± 0.1	8.1 ± 0.1	7.9 ± 0.2	0.28	0.99	0.83	0.29
Steps/day (n) ¹	4878 ± 240	5082 ± 165	16875 ± 463	<0.01*	0.62	<0.01*	<0.01*
Cadence (steps/min) ¹	85 ± 3	86 ± 2	94 ± 2	<0.01*	0.99	<0.01*	<0.01*
Estimated EE ¹ (MET·h/day)	32.0 ± 0.1	36.6 ± 0.2	37.0 ± 0.2	<0.01*	<0.01*	<0.01*	0.38

Abbreviations: SIT, sit regime; EXE, exercise regime; SL, sit less regime. Data are expressed as mean ± SE. N=12, ¹n=11. *p<0.05.

Insulin sensitivity

We examined the effects of the SIT, EXE and SL regimes on whole body and hepatic insulin sensitivity. To this end, insulin sensitivity was assessed by a two-step hyperinsulinemic-euglycemic clamp. Fasting plasma glucose levels measured before the start of the clamp were not different between the three activity regimes (SIT 5.4 ± 0.1, EXE 5.5 ± 0.1, SL 5.4 ± 0.1 mmol/l, p=0.29, **Figure 2A**). Whole body insulin sensitivity, as expressed by the change in glucose disposal (ΔR_d), was significantly higher in the EXE (28.2 ± 3.3 $\mu\text{mol/kg/min}$, p<0.01) and tended to be higher in the SL regimes (25.5 ± 2.8 $\mu\text{mol/kg/min}$, p=0.08) compared to the SIT regime (20.7 ± 3.7 $\mu\text{mol/kg/min}$, **Figure 2B** and **Supplementary Table 2**). The higher peripheral insulin sensitivity was mainly due to a significantly higher change in NODG (ΔNOGD) in the EXE regime (18.5 ± 2.6 $\mu\text{mol/kg/min}$, p=0.01) and a trend towards higher NOGD in the SL regime (15.0 ± 2.6 $\mu\text{mol/kg/min}$, p=0.10) compared to the SIT regime (12.2 ± 2.9 $\mu\text{mol/kg/min}$, **Figure 2C** and **Supplementary Table 2**). Carbohydrate oxidation during high insulin stimulation remained unchanged in the three activity regimes (SIT 13.8 ± 1.0, EXE 13.4 ± 1.1, SL 13.7 ± 1.2 $\mu\text{mol/kg/min}$, p=0.85). Hepatic insulin sensitivity, reflected by EGP suppression during the low insulin phase, was not

affected by the three activity regimes (SIT 73.6 ± 3.7 , EXE 69.2 ± 3.7 , SL 72.1 ± 2.9 %, $p=0.98$, Figure 2D and Supplementary Table 2).

Figure 2. Insulin sensitivity and intrahepatic lipid content

Insulin sensitivity assessed by a 2-step hyperinsulinemic-euglycemic clamp. **A:** Fasted plasma glucose, $n=11$. **B:** Whole body insulin stimulated glucose disposal expressed as the ΔR_d (high insulin minus baseline), $n=10$. **C:** Whole body non-oxidative glucose disposal expressed as the ΔNOGD (high insulin minus baseline), $n=10$. **D:** Suppression of hepatic endogenous glucose production expressed as the ΔEGP (low insulin minus baseline), $n=10$. **E:** Intrahepatic lipid content, $n=11$. Data are expressed as individual datapoints and mean $\pm$ SE. * $p<0.05$ and # $p<0.10$.

Intrahepatic lipid content

Intrahepatic lipid content remained similar between the three activity regimes (SIT 1.5 ± 0.3 , EXE 1.4 ± 0.3 , SL 1.3 ± 0.2 %, $p=0.91$, **Figure 2E**).

Substrate kinetics

Baseline RER tended to be lower in the SL regime compared to the SIT regime (SIT 0.78 ± 0.01 , EXE 0.77 ± 0.01 , SL 0.76 ± 0.01 , SIT vs SL $p=0.10$, **Figure 3C** and **Supplementary Table 2**). However, fat oxidation and CHO oxidation were not different between regimes ($p=0.19$ and $p=0.24$ respectively, **Figure 3A-B** and **Supplementary Table 2**). In addition, fat oxidation, CHO oxidation and RER during insulin stimulation were not affected by the activity regimes (**Supplementary Table 2**).

Figure 3. Baseline substrate oxidation

Baseline substrate oxidation measured by indirect calorimetry. **A:** Fat oxidation, $n=11$. **B:** Carbohydrate oxidation, $n=11$. **C:** Respiratory exchange ratio $n=11$. Data are expressed as individual datapoints and mean $\pm$ SE. # $p < 0.10$ between SIT and SL regime.

Mitochondrial respiration in skeletal muscle

We next investigated whether there would be a difference in skeletal muscle mitochondrial respiratory capacity between the three activity regimes. State 2 respiration, in the presence of substrate alone and without ATP synthesis, was not different between conditions (MO2: SIT 8.2 ± 0.6 , EXE 8.0 ± 0.4 , SL 8.0 ± 0.7 pmol/mg/s, $p=0.52$ and MG2: SIT 7.5 ± 0.7 , EXE 7.7 ± 0.5 , SL 6.9 ± 0.5 pmol/mg/s, $p=0.24$). Furthermore, ADP-stimulated (state 3) respiration upon complex I substrates (MG3) and upon lipid-derived substrates (MO3 and MOG3) were similar between the three activity regimes (MG3: SIT 37.5 ± 2.3 , EXE 38.5 ± 2.7 , SL 35.8 ± 2.2 pmol/mg/s, $p=0.42$, **Figure 4A**, MO3: SIT 36.1 ± 2.5 , EXE 37.7 ± 2.2 , SL 36.2 ± 2.9 pmol/mg/s, $p=0.66$, **Figure 4B**, MOG3: SIT 47.1 ± 3.9 , EXE 47.5 ± 2.6 , SL 44.7 ± 3.3 pmol/mg/s, $p=0.54$, **Figure 4C**). Similar results were observed upon parallel electron input to both complex I and II by sequentially adding succinate in both experiments (MGS3, $p=0.25$, **Figure 4D** and MOGS3, $p=0.58$, **Figure 4E**). Maximal FCCP-induced uncoupled respiration, reflecting the maximal capacity of the electron transport chain, was also unchanged (FCCP: SIT 88.5 ± 8.7 , EXE 93.7 ± 5.8 , SL 87.8 ± 7.2 pmol/mg/s, $p=0.53$, **Figure 4F**). Finally, addition of oligomycin, reflecting proton leak, was similar between the three activity regimes (Oligomycin: SIT 24.5 ± 1.8 , EXE 23.8 ± 1.5 , SL 23.0 ± 2.1 pmol/mg/s, $p=0.52$, **Figure 4G**).

Plasma triglycerides

Fasting plasma triglycerides were significantly lower in the EXE compared to the SIT regime (EXE 0.81 ± 0.12 , SIT 1.01 ± 0.15 mmol/l, $p=0.04$). No significant differences were observed between the SL and SIT regime (SL 0.83 ± 0.09 mmol/l, $p=0.13$) or the SL and EXE regime ($p=0.99$).

Blood pressure

Resting SBP and DBP, were not different between the three activity regimes (SBP: SIT 135 ± 4 , EXE 138 ± 6 , SL 135 ± 4 mmHg, $p=0.18$ and DBP: SIT 87 ± 2 , EXE 87 ± 3 , SL 87 ± 2 mmHg, $p=0.91$). Resting HR was similar between the activity regimes as well (SIT: 57 ± 1 , EXE 56 ± 2 , SL 56 ± 2 bpm, $p=0.37$).

Figure 4. *Ex vivo* skeletal muscle mitochondrial respiratory capacity

State 3 coupled respiration upon **A**: malate + glutamate (MG3), **B**: malate + octanoyl carnitine + glutamate (MO3), **C**: malate + octanoyl carnitine + glutamate (MOG3), **D**: malate + glutamate + succinate (MGS3) and **E**: malate + octanoyl carnitine + glutamate + succinate (MOGS3). **F**: Maximal FCCP-induced uncoupled respiration. **G**: Oligomycin induced respiration not coupled to ATP synthesis. All $n=11$. Data are expressed as individual datapoints and mean $\pm$ SE.

DISCUSSION

A number of studies investigated the reduction in sedentary behaviour and showed improvements in several aspects of metabolic health, including a better glucose homeostasis (9-16). Sitting time can be replaced by light activities such as standing and walking or by moderate-to-vigorous physical activity. Both strategies have been proven effective in improving glucose homeostasis (9-16). However, the underlying mechanisms and whether these two sitting replacement strategies act via similar mechanisms remain to be investigated. Here, we hypothesized that replacing sitting time with standing and walking would exert improvements in glucose homeostasis via an increase in peripheral and hepatic insulin sensitivity. Furthermore, we hypothesized that the improvement in insulin sensitivity could coincide with an increase in muscle mitochondrial function and a reduction in intrahepatic lipid content. To investigate these hypotheses, we performed a randomized three-arm cross-over intervention study in which participants underwent three times a 4-day activity regime – sit regime, exercise regime and sit less regime – after which insulin sensitivity, muscle mitochondrial function and intrahepatic lipid content were assessed.

The activity regimes were successfully implemented as reflected by the significant differences in standing, walking, sitting and exercising time between the three activity regimes, as anticipated. By design, the exercise and sit less regimes had similar estimated daily energy expenditure. The amount of walking and standing time was kept equal in the sit and exercise regimes. This resulted in comparable sitting time between the sit and exercise regimes, and a comparable physical activity level between the sit less and exercise regimes. Thus, the intervention was successfully reproduced, as previously described by Duvivier et al. (14).

Our primary aim was to investigate the effects of the sit less and exercise regimes on insulin sensitivity compared to the sit regime. Several studies have shown improvements of reducing sedentary behaviour on glucose homeostasis measured by an oral glucose tolerance test (14-16) or by attenuated postprandial plasma glucose and insulin responses during meal tests (9-13). While oral glucose tolerance tests and meal tests give valuable information about glucose homeostasis, the gold standard for assessing human insulin sensitivity is the hyperinsulinemic-euglycemic clamp developed by DeFronzo et al (34). So far, this technique has only been used once to investigate the effects of sedentary behaviour on peripheral insulin sensitivity (35). Thus, Krogh et al. (35) applied a more extreme intervention in which healthy young participants reduced their walking activity from an habitual ~10500 steps per

day to ~1400 steps per day for 2 weeks, which resulted in a decrease in peripheral insulin sensitivity (expressed as the glucose infusion rate) of ~17%. However, they did not compare a sit less regime with an exercise regime. Here, we applied the 2-step hyperinsulinemic-euglycemic clamp technique in combination with glucose tracer, enabling us to discriminate between peripheral and hepatic insulin sensitivity. In line with our hypothesis, we report a significant increase in peripheral insulin sensitivity with the exercise regime (~33%) and nearly significant increase with the sit less regime (~22%), both compared to the sit regime. The increase in insulin-stimulated peripheral glucose uptake could be assigned to an increase in insulin-stimulated glycogen storage (NOGD) rather than glucose oxidation. No effects of any of the regimes were detected on hepatic insulin sensitivity. Therefore, based on our results we can conclude that the sit less and the exercise regimes were both effective in improving peripheral insulin sensitivity, suggesting that reducing sitting time forms a potential effective strategy in the prevention or treatment of type 2 diabetes.

It has previously been shown by us (36) and others (37) that skeletal muscle insulin resistance is associated with a reduction in mitochondrial function. Therefore, to investigate if the beneficial effects of sit less and exercise were due to enhancement of mitochondrial capacity, we measured *ex vivo* skeletal muscle mitochondrial respiratory capacity upon reducing sedentary behaviour by standing and walking, and upon exercise. However, in contrast to our hypothesis, the observed improvement in peripheral insulin sensitivity could not be explained by an increase in muscle mitochondrial function. In addition, no effect on muscle mitochondrial function was found after the exercise regime either. Although it is generally accepted that exercise exerts positive effects on muscle mitochondrial function and biogenesis (19, 38), and that this partly underlies long term training-induced improvements seen in peripheral insulin sensitivity (37, 39), the duration of the current study (four days) may have been too short to detect such effects (40). Together, these data indicate that the beneficial effects of short-duration exercise or sit less regimes are independent of mitochondrial changes and other mechanisms must be underlying. To further unravel these, muscle biopsies will be used to study GLUT4 translocation and for metabolomics analyses, and these analyses are currently ongoing.

Besides an improvement in peripheral insulin sensitivity, fasted plasma triglyceride levels are affected by physical activity as well. We confirm the previously reported (14-16) reduction in fasted plasma triglyceride levels upon the exercise regime compared to the sit regime. Although not confirmed by us and some others (9, 12), it has in addition been shown that replacing sedentary behaviour by light intensity

physical activities was successful as well in reducing fasted plasma triglycerides (14-16). Nonalcoholic fatty liver disease is associated with increased plasma triglyceride levels (41) and high intrahepatic lipid content has shown to be an independent contributor to obesity-induced insulin resistance (42). Moreover, higher intrahepatic lipid content has been positively correlated with sedentary time in a large cohort study (43), although another study did not report such a relation (44). Therefore, we investigated whether intrahepatic lipid content was reduced upon the sit less and exercise regimes compared to the sit regime and could play a role in changes in insulin sensitivity. In contrast to our hypothesis, intrahepatic lipid content was not affected by the three activity regimes and the lack of change in intrahepatic lipid content was reflected by an absent change in hepatic insulin sensitivity. This finding contrasts the reduction in intrahepatic lipid content found upon a prolonged resistance and endurance exercise training (45), and may indicate that a four-day intervention is too short to detect alterations in intrahepatic lipid content. Furthermore, these findings indicate that the main effects of sitting less resided to peripheral insulin sensitivity. In that respect, it has been reported that a reduced triglyceride level can also be caused by enhanced clearance of triglycerides in the muscle via stimulated lipoprotein lipase activity (46, 47). Indeed, in rodents, it has been shown that muscle lipoprotein lipase activity is low upon inactivity and already increased upon low-intensity muscle contractions, in this case slowly walking (48). Further analysis in muscle biopsies are needed to reveal if LPL activity and triglyceride clearance in muscle was elevated upon sit less.

In conclusion, we here show that replacing sedentary time by light physical activities, such as standing and walking, is nearly as effective in improving peripheral insulin sensitivity as replacing sedentary time by a single vigorous exercise bout per day. The improvement in peripheral insulin sensitivity could however not be explained by an increase in muscle mitochondrial function. Moreover, hepatic insulin sensitivity and intrahepatic lipid content were not affected by reducing sedentary behaviour or exercise during this four-day intervention strategy. Given the more prominent role of reducing sedentary behaviour in the prevention and treatment of type 2 diabetes nowadays, future research should evaluate the practical applicability of such a sit less regime in daily life.

SUPPLEMENTARY MATERIAL FOR CHAPTER 5

Supplementary Table 1. Medication of participants

Medication	N
Lipid lowering drugs (statines)	2
Blood pressure lowering drugs	1
Glaucoma medication	1

Supplementary Table 2. Insulin sensitivity and substrate kinetics

Parameter	SIT	EXE	SL	p-value	p-value SIT vs EXE	p-value SIT vs SL	p-value EXE vs SL
Ra ($\mu\text{mol/kg/min}$)							
Baseline	7.6 $\pm$ 0.4	7.3 $\pm$ 0.5	8.4 $\pm$ 0.5	0.21	0.99	0.46	0.31
Low insulin ¹	11.9 $\pm$ 1.3	11.7 $\pm$ 1.0	11.9 $\pm$ 0.7	0.73	0.99	0.99	0.99
High insulin ²	29.8 $\pm$ 3.6	36.1 $\pm$ 3.8	32.8 $\pm$ 3.2	<0.01**	0.01*	0.22	0.79
Rd ($\mu\text{mol/kg/min}$)							
Baseline	7.3 $\pm$ 0.4	6.9 $\pm$ 0.5	7.6 $\pm$ 0.6	0.56	0.99	0.99	0.92
Low insulin ¹	12.5 $\pm$ 1.2	11.8 $\pm$ 0.9	12.3 $\pm$ 0.9	0.73	0.99	0.99	0.99
High insulin ²	29.4 $\pm$ 3.7	35.2 $\pm$ 3.8	33.1 $\pm$ 3.2	<0.01**	<0.01**	0.03*	0.99
Delta baseline-low ¹	5.0 $\pm$ 1.2	4.9 $\pm$ 0.8	4.7 $\pm$ 0.7	0.98	0.99	0.99	0.99
Delta baseline-high ¹	20.7 $\pm$ 3.7	28.2 $\pm$ 3.3	25.5 $\pm$ 2.8	<0.01**	<0.01**	0.08#	0.99
EGP ($\mu\text{mol/kg/min}$)							
Baseline	7.6 $\pm$ 0.4	7.3 $\pm$ 0.5	8.4 $\pm$ 0.5	0.21	0.99	0.46	0.31
Low insulin ¹	2.0 $\pm$ 0.2	2.3 $\pm$ 0.2	2.3 $\pm$ 0.2	0.16	0.60	0.17	0.99
% suppression low ¹	73.6 $\pm$ 3.7	69.2 $\pm$ 3.7	72.1 $\pm$ 2.9	0.98	0.99	0.99	0.99
High insulin ²	0.4 $\pm$ 0.2	0.7 $\pm$ 0.2	0.3 $\pm$ 0.1	0.71	0.99	0.99	0.99
% suppression high ²	93.6 $\pm$ 2.7	90.0 $\pm$ 2.9	96.4 $\pm$ 1.9	0.44	0.99	0.99	0.54
NOGD ($\mu\text{mol/kg/min}$)							
Baseline ¹	2.6 $\pm$ 0.6	3.0 $\pm$ 0.6	4.0 $\pm$ 0.7	0.63	0.99	0.99	0.86
Low insulin ¹	3.9 $\pm$ 1.2	4.3 $\pm$ 0.9	4.9 $\pm$ 1.0	0.63	0.86	0.99	0.99
High insulin ²	15.7 $\pm$ 2.8	22.3 $\pm$ 3.0	19.9 $\pm$ 2.6	<0.01**	<0.01**	0.04*	0.79
Delta baseline-low ²	1.0 $\pm$ 1.7	1.0 $\pm$ 0.8	1.0 $\pm$ 0.7	0.60	0.99	0.79	0.99
Delta baseline-high ³	12.2 $\pm$ 2.9	18.5 $\pm$ 2.6	15.0 $\pm$ 2.6	0.01*	0.01*	0.10#	0.99
Carbohydrate oxidation ($\mu\text{mol/kg/min}$)							
Baseline ¹	5.0 $\pm$ 0.6	4.5 $\pm$ 1.0	4.2 $\pm$ 0.7	0.24	0.46	0.13	0.91
Low insulin ¹	8.6 $\pm$ 0.6	7.5 $\pm$ 0.8	7.5 $\pm$ 0.6	0.35	0.41	0.86	0.99
High insulin ²	13.8 $\pm$ 1.0	13.4 $\pm$ 1.1	13.7 $\pm$ 1.2	0.98	0.99	0.99	0.99
Fat oxidation ($\mu\text{mol/kg/min}$)							
Baseline ¹	3.3 $\pm$ 0.2	3.7 $\pm$ 0.2	3.6 $\pm$ 0.1	0.19	0.33	0.18	0.81
Low insulin ¹	2.3 $\pm$ 0.2	2.5 $\pm$ 0.2	2.6 $\pm$ 0.2	0.26	0.26	0.99	0.99
High insulin ²	1.1 $\pm$ 0.2	1.3 $\pm$ 0.2	1.3 $\pm$ 0.2	0.51	0.99	0.72	0.99
Respiratory exchange ratio							
Baseline ¹	0.78 $\pm$ 0.01	0.76 $\pm$ 0.01	0.76 $\pm$ 0.01	0.16	0.59	0.10#	0.99
Low insulin ¹	0.84 $\pm$ 0.01	0.82 $\pm$ 0.01	0.82 $\pm$ 0.01	0.20	0.63	0.22	0.99
High insulin ²	0.92 $\pm$ 0.01	0.91 $\pm$ 0.01	0.91 $\pm$ 0.01	0.63	0.99	0.99	0.99
Delta baseline-low	0.07 $\pm$ 0.01	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01	0.66	0.99	0.99	0.99
Delta baseline-high	0.13 $\pm$ 0.01	0.14 $\pm$ 0.02	0.14 $\pm$ 0.02	0.69	0.99	0.99	0.99

Abbreviations: SIT, sit regime; EXE, exercise regime; SL, sit less regime; Ra, rate of appearance; Rd, rate of disappearance; EGP, endogenous glucose production; NOGD, non-oxidative glucose disposal. Data are expressed as mean $\pm$ SE. ¹n=11, ²n=10, ³n=9. ** p<0.01, *p<0.05, #p<0.10.

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CHAPTER 6

General discussion and conclusion

Over the last decades, the worldwide prevalence of overweight, obesity and type 2 diabetes mellitus (T2DM) has increased dramatically (1, 2). The fundamental cause of the increased prevalence is an unhealthy lifestyle leading to a sustained positive energy balance, in which energy intake exceeds energy expenditure for a prolonged period of time, with body mass – mostly fat mass – gain as a consequence. Obesity and excessive fat mass can lead to impaired insulin sensitivity and mitochondrial dysfunction, which are key characteristics of metabolic diseases such as T2DM. Lifestyle interventions like caloric restriction and exercise training are well known strategies to counteract this positive energy balance and to improve markers of human metabolic health, such as insulin sensitivity. The first demonstration of the beneficial health effects of dietary caloric restriction – without malnutrition – was already reported in the 1930s (3). While obesity was not a major problem at that time, caloric restriction was reported as a method for extending lifespan and slowing aging in a variety of species, while maintaining health and vitality. Further research in rodents (4-6) and primates (7, 8) showed an improvement in glucose homeostasis – reflected in an increased insulin sensitivity – and an enhanced biogenesis of mitochondria. These improvements in insulin sensitivity and mitochondrial biogenesis were considered important underlying metabolic processes in mediating the physiological health effects of caloric restriction (9). In more recent years, the effects of caloric restriction have also been studied in overweight humans in whom the health effects of a 25% reduction in energy intake for six months were evaluated (10-12). These studies observed that caloric restriction is an effective strategy to increase human insulin sensitivity and mitochondrial function, alongside improvements in other markers of metabolic health (10-12).

The effects of caloric restriction are among others mediated via downstream signals sensing a low cellular energy status, leading to activation of AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1), and via weight loss. An alternative strategy to induce cellular energy stress is via exercise training. Exercise training is not necessarily accompanied by weight loss but does increase energy expenditure and thereby also activates downstream signals of energy stress. Exercise training involves muscle contractions, which activates translocation of the glucose transporter (GLUT4) to the muscle cell membrane to facilitate glucose uptake. Exercise-mediated translocation of GLUT4 is triggered by activation of AMPK as well. For several decades, research has been performed to study the health effects of exercise training. Beneficial effects of various exercise training programs have emerged, including improvements in glucose homeostasis, insulin sensitivity and mitochondrial function (13-18). Moreover, results conclude that exercise training is beneficial for T2DM, as it beneficially influences incidence and prognostic rates (19).

Taken together, clear improvements in human metabolic health can be achieved via inducing a low cellular energy status by caloric restriction and via activation of skeletal muscle by exercise training. Indeed, both strategies reduce the risk of developing metabolic diseases, such as T2DM (19, 20). Although caloric restriction and exercise training are very powerful, the practical applicability is less good; long-term compliance to a low-calorie-diet and regular exercise training is very low in the overweight and obese population who would benefit the most from these lifestyle changes (21, 22). Therefore, research is needed to find alternative and more readily acceptable ways to improve human metabolic health. The obtained knowledge regarding the underlying mechanisms of caloric restriction and exercise training (thus, activation of AMPK, SIRT1, and GLUT4 translocation) are useful to generate new hypothesis and to discover and design new strategies to combat metabolic diseases such as T2DM. In this thesis, the hypothesis that activation of skeletal muscle metabolism and promotion of whole-body cellular energy metabolism trigger a range of beneficial metabolic adaptations has been tested using multiple novel intervention strategies. The primary aim of this thesis was to target human insulin sensitivity and muscle mitochondrial function to improve human metabolic health.

SKELETAL MUSCLE ACTIVATION: PHYSICAL ACTIVITY

Exercise training refers to structured, planned, and repetitive physical activities with the aim to improve or maintain physical fitness (23). However, any bodily movement produced by skeletal muscles requires energy and can be described as physical activity (23). From epidemiological studies it is known that people who are more physically active display a better metabolic health, reflected by lower risk markers for T2DM and cardiovascular diseases, than people who are less physically active (19, 24-26). Importantly, high physical activity levels have been associated with a reduced mortality risk (19, 27). In recent years, not only the amount of time spent exercising, but also the amount of time spent sedentary has been associated with T2DM, the metabolic syndrome (28, 29), and increased mortality risk (30). A recently published review highlighted that physical inactivity – including short-term inactivity – induces peripheral insulin resistance in skeletal muscle and adipose tissue with consequent accumulation of fat in the liver, leading to hepatic insulin resistance (31). Fortunately, it has also been suggested that the effects of physical inactivity are reversible (31). Therefore, research has been performed on different strategies to reduce sedentary behaviour.

Sedentary time can be replaced by moderate-to-vigorous physical activities such as exercise training or by light intensity physical activities such as standing and walking. Whether sedentary time should be replaced by exercise training or by standing and

walking to obtain maximal benefit, is still under debate. Several human intervention studies in which sedentary time was replaced by standing (32, 33), walking (32-36) or short exercise bouts (36) have all shown improvements in glucose homeostasis. In general, standing and walking is more feasible for a large proportion of the obese population for whom exercise compliance generally is low. Therefore, it needs to be investigated whether these sitting replacement strategies possess beneficial health effects. It has previously been observed that a four-day intervention, in which replacement of sedentary time by 3 hours of standing and 2 hours of walking, or by a single 1-hour exercise-bout per day, both improved glucose and lipid metabolism, including markers for whole-body insulin sensitivity (37-39). In three different studies, these effects were reported in healthy young adults, healthy obese men and women and in patients with T2DM (37-39). Furthermore, the effects were more pronounced in women (37). Results indicate that sedentary time indeed can be replaced by light intensity physical activities such as standing and walking to improve metabolic health parameters. The question which remains to be answered is whether these two sitting replacement strategies (standing/walking vs. exercise) act via similar or different underlying pathways involving skeletal muscle.

In **chapter 5**, we show that replacing sedentary time by several hours of standing and walking (sit less regime) was similar effective in improving human peripheral – mainly skeletal muscle – insulin sensitivity compared to a single moderate-to-vigorous exercise bout per day (exercise regime) in healthy obese women. These results are in agreement with previous studies which reported improvements in markers of whole-body insulin sensitivity after similar interventions, measured by an oral glucose tolerance test and by 24-hour glucose monitoring (37-39). We examined insulin sensitivity by a 2-step hyperinsulinemic euglycemic clamp combined with a glucose tracer, which is the gold standard to assess peripheral and hepatic insulin sensitivity (40). We show that hepatic insulin sensitivity and also hepatic lipid content were not affected by the four-day intervention (**chapter 5**), which means that merely muscle was responsible for the exerted improved insulin-stimulated glucose uptake.

Improvements in insulin sensitivity after exercise training are reported together with an increased muscle mitochondrial function and muscle mitochondrial content (15). Therefore, we collected muscle tissue in our study and measured muscle mitochondrial respiratory capacity to investigate whether the beneficial effects of the four-day sit less regime and the four-day exercise regime on peripheral insulin sensitivity involved improvements in muscle mitochondrial function. However, we found that neither the sit less nor the exercise regime affected skeletal muscle mitochondrial function compared to the sit regime (**chapter 5**). Acute exercise can

also increase insulin sensitivity independent of effects on mitochondrial function or hepatic metabolism. As explained previously, acute exercise activates pathways in skeletal muscle in response to changes in energy status. This in turn leads to activation of AMPK and subsequent GLUT4 translocation, which stimulates glucose uptake in muscle and increases insulin sensitivity (41). These effects remain several hours after the final exercise-bout and involve enhanced glucose uptake in the liver to replenish glycogen stores (41). Therefore, our results suggest that short-term improvements in peripheral insulin sensitivity induced by standing and walking probably act via similar pathways in skeletal muscle as acute exercise training. It has, however, been shown that longer term exercise training interventions do report positive effects regarding muscle mitochondrial function and hepatic lipid content after for example 12 weeks of training (14, 16). Thus, we cannot rule out that longer-term interventions in which sedentary time is replaced by standing and walking can also improve muscle mitochondrial function and hepatic metabolism. In the near future, muscle tissue sampled in the present study will be studied using transcriptomics and metabolomics to identify early signs of metabolic adaptations following the sit less regime in comparison to the exercise regime.

SKELETAL MUSCLE ACTIVATION: SHIVERING

An alternative way to increase energy expenditure is by cold exposure. Physiological responses to cold exposure include insulative responses by peripheral vasoconstriction and metabolic responses by increasing energy expenditure with the aim to produce heat and to maintain core body temperature stable. Heat is a side product of energy expenditure, both when work is performed, but also when energy is produced to fuel vital bodily functions. This thermogenic metabolic response can be divided into shivering thermogenesis and non-shivering thermogenesis. Shivering thermogenesis is a physiological reaction of the human body to acute cold exposure and includes involuntary muscle contractions. Non-shivering thermogenesis increases metabolic rate without activating muscle shivering, and brown adipose tissue (BAT) plays a major role herein. It has been reported that before the onset of shivering, metabolic rate already increases indicating that non-shivering thermogenesis precedes shivering (42). BAT is a special form of adipose tissue, characterized by a large number of mitochondria that contain the uncoupling protein UCP1, which puts BAT mitochondria in the position to generate heat rather than ATP. BAT-based non-shivering thermogenesis has an evolutionary advantage during times when heat loss is massive, such as in newborn and small mammals, who are prone to hypothermia due to their high surface-to-body ratio. The relevance of BAT in adult humans has long been questioned, since the absolute amount (and hence its capacity for thermogenesis) is considered to be very low in adult humans. However,

it has been shown that BAT activity and thermogenesis is negatively correlated with body fat mass in humans (43). Therefore, a relationship was proposed between the increase in the prevalence of obesity and the fact that many people nowadays live in their thermoneutral zone for most of the day, thus, there is no need to increase energy expenditure for thermal comfort (44). Therefore, re-activating BAT is a way to promote energy expenditure and thereby could contribute to changes in body mass.

In the last decade, BAT has received much scientific attention as the major side of non-shivering thermogenesis, and it has been investigated whether increasing BAT activity or volume could protect against obesity and related metabolic diseases such as T2DM. Indeed, studies in adult humans have shown that acute cold exposure activates BAT and increases whole body energy expenditure (45-48). In **chapter 3** we confirm that acute mild cold exposure just below the individual shivering threshold increases BAT activity and resting energy expenditure in healthy obese humans.

Interestingly, in recent years it has been reported several times that cold exposure may also affect insulin sensitivity (46, 49-51) and that these effects may be in the same order of magnitude as exercise training (51). Originally, activation of BAT was the proposed mechanism of action. More recently, however, it has been shown that cold acclimation mainly affects peripheral insulin sensitivity via an effect on skeletal muscle GLUT4 translocation (51). Indeed, both skeletal muscle (52, 53) and BAT (43, 47, 54, 55) contribute to non-shivering thermogenesis during cold acclimation. Muscle-based non-shivering thermogenesis is energetically costly (56) and thereby can contribute to a higher energy expenditure as well. Considering that more than 40% of total body mass is represented by skeletal muscle, and that multiple non-shivering thermogenesis mechanisms have been identified within skeletal muscle (mitochondrial uncoupling, protein turnover, fatty acid cycling, and calcium cycling (57)), it is capable of producing a significant amount of heat and thereby contributing to whole body thermogenesis. However, although it is suggested that muscle-based non-shivering thermogenesis might be a target to improve metabolic health (51), part of the effects of cold acclimation on insulin sensitivity may also be explained by shivering thermogenesis.

When skin and core temperature decrease, shivering intensity increases (58, 59). Heat production through shivering thermogenesis can reach maximal values equivalent to around five times resting metabolic rate (60). In comparison, high intensity exercise can increase resting metabolic rate up to 20 times (61). Interestingly, upon acute cold exposure to 5 °C in humans, fat oxidation and carbohydrate oxidation increase to mobilize fuels for thermogenesis (62). Moreover,

acute cold exposure to 10 °C in humans may promote insulin sensitivity, an effect that predominantly originates from muscles that actually are shivering (63). These data suggest that part of the effect of cold exposure on insulin sensitivity could be due to a stimulation of shivering thermogenesis.

Previous research performed within our department reported an increase of approximately 40% in peripheral insulin sensitivity upon ten days of mild cold acclimation (14-15 °C) (51). The reported increase in peripheral insulin sensitivity was accompanied by increased translocation of GLUT4 to the cell membrane in skeletal muscle. Given the relative mild cold exposure and the attempt to minimize shivering, it was suggested that this improvement in insulin sensitivity was due to a combination of non-shivering thermogenesis in BAT and skeletal muscle (51). To further investigate this, in **chapter 4**, we performed a study with a similar study design (ten days of mild cold acclimation in overweight and obese patients with T2DM), except with a slightly higher temperature (16-17 °C) and with more detailed attention for the prevention of shivering. The aim of the study was to investigate whether the cold-induced improvements in peripheral insulin sensitivity (as observed before (51)) were sustained for a longer period of time after cessation of the cold intervention, and whether this was accompanied by improvements in postprandial glucose and lipid metabolism and markers of cardiovascular health. Unexpectedly, we could not reproduce the improvements in peripheral insulin sensitivity upon mild cold exposure and hence also no improvements in the other outcomes were observed (**chapter 4**). Given the focus on non-shivering thermogenesis and the somewhat higher temperature during cold exposure in our study, we investigated whether the two studies could be distinguished from each other by the presence or absence of shivering. Comparison of questionnaire results showed that some mild form of shivering occurred in the previous study (51) compared to almost no shivering in our current study (**chapter 4**). Subsequently, analyses in skeletal muscle biopsies were performed to evaluate GLUT4 translocation and gene expression of markers for skeletal muscle contraction. In contrast to the previous study (51), we did not show any translocation of GLUT4 to the cell membrane after the cold acclimation, matching with the absent improvement in peripheral insulin sensitivity in our current study (**chapter 4**). In addition, we performed gene-expression analysis in skeletal muscle biopsies obtained before and after cold acclimation in the previous and current study. Genes were selected from micro-array data obtained after the previous cold acclimation study (51) compared to an exercise training study (14), to function as markers of muscle contraction. The selected genes revealed overlap in skeletal muscle gene expression-patterns related to muscle contraction pathways

(unpublished data). Thus, these muscle contraction markers were increased in the previous study, suggesting that shivering may have occurred in the previous study (51). Interestingly, no change in skeletal muscle gene expression was observed in the current study (**chapter 4**). Therefore, our results indicate that modest shivering or increased muscle tension may be the underlying pathway through which improvements in peripheral insulin sensitivity and GLUT4 translocation were observed upon ten days mild cold acclimation in the previous study (51), but not in our current study (**chapter 4**). Further research is needed to quantify the amount of shivering that is needed to induce metabolic changes, which can for example be done by electromyographic activity measurements. Also, metabolic pathways that may be activated by this mild shivering, such as AMPK activation, need to be studied to understand how mild shivering is connected to an improvement in skeletal muscle insulin sensitivity.

PROMOTING CELLULAR ENERGY AND NAD⁺ METABOLISM

Besides the investigation of several strategies to promote skeletal muscle activity, another strategy is to search for compounds that can mimic the beneficial effects of caloric restriction by promoting energy metabolism without the need to change lifestyle. To be effective, a caloric restriction mimetic should alter the key metabolic pathways. Thus, targeting SIRT1 activation either directly or indirectly via AMPK may result in similar beneficial effects as observed after caloric restriction. Several caloric restriction mimetics have been studied in the past years. The oral antidiabetic drug metformin is the most described one and is thought to mediate its anti-hyperglycemia and insulin-sensitizing effects via AMPK and SIRT1 activation (64, 65). Another example is the nutritional compound resveratrol, which activates AMPK and SIRT1 (66, 67) and has been reported to improve human skeletal muscle mitochondrial capacity after 30 days supplementation (66, 68, 69). Furthermore, resveratrol and other polyphenols, such as quercetin, reduce reactive oxygen species production and increase mitochondrial capacity (70). Mitochondrial capacity can be improved by enhancing mitochondrial biogenesis, but also by enhancing mitophagy and thus improving mitochondrial quality via increasing mitochondrial turnover. Urolithin A is an example of a nutritional compound that stimulates mitophagy and has recently been shown to induce a molecular signature in human skeletal muscle of improved mitochondrial and cellular health (71). These examples show that several ways of targeting SIRT1 activity and cellular energy metabolism have been studied and can be used to stimulate mitochondrial metabolism in humans.

One key metabolite in the signaling of cellular energy status that may link stimulation of energy metabolism to improvement in mitochondrial function and metabolic health

is nicotinamide adenine dinucleotide (NAD⁺). NAD⁺ is a critical signaling molecule of cellular energy status and is an essential substrate for the NAD⁺-dependent enzyme SIRT1. As previously explained, SIRT1 is a metabolic regulator of oxidative energy metabolism and is activated in situations of energy stress like exercise and caloric restriction (72). Furthermore, caloric restriction and exercise training are known to upregulate the enzyme nicotinamide phosphoribosyl transferase (NAMPT), which is involved in NAD⁺ synthesis (73, 74). NAMPT levels correlate positively with skeletal muscle mitochondrial function and overall aerobic capacity (73). Since NAD⁺ levels are decreased in the obese and older population (75-77), replenishment of NAD⁺ levels is hypothesized to be a new strategy to improve human metabolic health, and is suggested to have more profound effects compared to other nutritional compounds such as resveratrol. NAD⁺ levels can be increased via dietary supplementation of NAD⁺-precursors or via inhibition of NAD⁺ degradation pathways, or both.

In humans, several NAD⁺ precursors have already been tested. Thus, nicotinic acid and acipimox are effective in lowering circulating lipids, but due to a rebound effect, fatty acids in the circulation increase on the longer term with increased muscle lipid content, insulin resistance, and elevated plasma glucose levels as consequences (78, 79). Nicotinamide is another factor that has been shown to increase NAD⁺ levels, however, nicotinamide has not been reported to have beneficial metabolic health effects so far, and high dosages are hepatotoxic (80). However, all these three NAD⁺-precursors, have been shown to induce unwarranted side effects in humans by binding to the G protein-coupled receptor, GPR109A, and thereby inducing flushing (81, 82). Nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) – second generation NAD⁺-precursors – do not induce these side effects and are orally bioavailable endogenous molecules. This renders NMN and NR the molecules of choice for human trials nowadays. In this thesis, the effects of NR on human metabolic health has been investigated.

So far, NAD⁺ replenishment by nutritional supplementation with NR has been successful in improving insulin sensitivity and other parameters of metabolic health in animal models (75, 83-86). Within these animal studies, it has been shown that NR is taken up in several tissues including skeletal muscle, liver, heart and brown adipose tissue, reflected by increased levels of NAD⁺ within these tissues (83). Whether NR is also taken up in white adipose tissue is still inconclusive (83, 86). Clinical trials in humans are warranted to test the translational value of the results from this animal research. Therefore, in this thesis, the effects of six weeks NR supplementation in healthy overweight and obese humans have been investigated on whole body energy metabolism and on specific tissues. In **chapter 2** we show that upon NR

supplementation, markers of enhanced NAD⁺ metabolism in skeletal muscle are induced. No change in skeletal muscle NAD⁺ content was detected, however. Although inconsistent with animal data on NR supplementation (83, 84), our results are in agreement with other data from human intervention studies that investigated the effects of NR supplementation on skeletal muscle NAD⁺ metabolism and reported increased NAD⁺ metabolites (NAAD and MeNAM) as well (87, 88). Furthermore, in our and previous studies, no positive effects on skeletal muscle mitochondrial respiration, peripheral insulin sensitivity or intramyocellular lipid content were observed (87-89) (**chapter 2**).

These results indicate that NR does not have the hypothesized effects on human skeletal muscle metabolism. Furthermore, our results indicate that skeletal muscle NAD⁺ turnover is increased, which is not reflected in steady state NAD⁺ levels in skeletal muscle. Results in **chapter 2** however support the idea that NR indeed may have modest effects on human skeletal muscle, as an increase in fat free mass – which mainly represents muscle mass – upon NR supplementation was observed. The increase in muscle mass might help to understand the observed increase in sleeping metabolic rate after NR supplementation. Moreover, skeletal muscle acetylcarnitine levels were affected by NR supplementation. The role of acetylcarnitines in regulating i.e. fatty acid oxidation and the role of NR herein needs further investigation. Future studies should be designed to reproduce if NR supplementation can indeed increase muscle mass and affect acetylcarnitine levels in humans and to investigate the underlying mechanisms. Besides these modest effects of NR on human skeletal muscle metabolism, no other metabolic effects were observed on whole body substrate oxidation (**chapter 2**) or in other tissues, such as intrahepatic lipid content, cardiac energy status (**chapter 2**) or brown adipose tissue activity (**chapter 3**). While, we were successful in reproducing the earlier reported (83) stimulatory effects of NR in high fat diet-fed mice on BAT activity in **chapter 3**, again no effects of NR on human BAT *in vivo* were shown (**chapter 3**). Further research is needed to evaluate various dosages and long-term supplementation to investigate if NR can have effects on human metabolic health.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Research described in this thesis aimed to investigate novel strategies to improve human metabolic health with the main focus on insulin sensitivity and muscle mitochondrial function. Over the past decades it has become clear that caloric restriction and exercise training are successful strategies to improve human metabolic health, including insulin sensitivity and mitochondrial function, and that this leads to a reduction in the risk on developing T2DM and cardiovascular diseases. It

has been confirmed in this thesis that physical activity is a powerful tool to increase peripheral insulin sensitivity, already after a four-day intervention. Moderate-to-vigorous physical activities such as exercise, as well as light-intensity physical activities such as standing and walking, promoted peripheral insulin sensitivity to a similar extent in healthy obese women (**chapter 5**). The aim of this four-day intervention study was to investigate whether similar pathways were responsible for this effect. So far, similar metabolic routes were observed between the two sitting replacement strategies in outcomes regarding peripheral and hepatic insulin sensitivity, muscle mitochondrial function, and hepatic lipid content. Completion of this study by performing analysis in the obtained skeletal muscle tissue should reveal more information about underlying molecular pathways activated by standing and walking versus exercise. Pathways of interest to measure include GLUT4 translocation, insulin signaling, and calcium cycling since these pathways play a role in insulin sensitivity and cellular energy status. Furthermore, the determination of triglyceride clearance, lipoprotein lipase activity, and intramyocellular lipid storage would be of interest as well, since we (**chapter 5**) and others (32, 33, 39, 90) report lower triglyceride levels after short-term exercise training, and some studies also after a standing and walking intervention (37-39, 91). The long-term effects of a sit less regime should be investigated, as this could shed more light on the underlying pathways in skeletal muscle. Lastly, dose-response studies should be performed to evaluate the optimal intensity, duration and pattern of sedentary interruptions to provide information for public recommendations.

From another point of view, physical activity in the form of shivering might be a successful strategy to increase peripheral insulin sensitivity as well. Results presented in **chapter 4** provide evidence that shivering thermogenesis might be an important underlying mechanism through which improvements in peripheral insulin sensitivity after ten days of mild cold exposure have been reported previously (51). Although acute mild cold exposure without shivering is able to increase whole body energy metabolism via BAT activation in healthy obese humans (**chapter 3**), this cold stimulus does not seem to be powerful enough to also induce changes in skeletal muscle metabolism of patients with T2DM after 10 days of mild cold acclimation (**chapter 4**). Future studies should investigate underlying metabolic pathways that may be activated by (mild) shivering – for example AMPK activation – to understand how shivering is linked to improvements in skeletal muscle insulin sensitivity. Furthermore, the minimal intensity and duration of shivering that exerts sustained beneficial metabolic health effects must be identified.

Our third novel strategy was to improve human metabolic health by promoting NAD⁺ metabolism. Results from this thesis illustrate that there is limited evidence that six

weeks of supplementation with the NAD⁺ precursor NR is effective in improving markers of metabolic health in healthy obese humans (**chapter 2 and 3**). The results obtained in our study and by other human clinical trials (87-89) do not support the hypothesis that NR supplementation is beneficial in improving muscle mitochondrial function and insulin sensitivity in humans. However, it does not mean that further research is not warranted. Interestingly, we and others (87, 88) do show some effects of NR supplementation on elevating NAD⁺ metabolite levels in skeletal muscle and in addition we show some minor effects in skeletal muscle metabolism regarding fat free mass and acetylcarnitine levels (**chapter 2**). Further research is needed to investigate the exact link between NR metabolism and acetylcarnitine metabolism. Furthermore, as we and others only investigated the effects of NR supplementation in a middle-aged, obese, healthy population, we cannot exclude the possibility that NR would have effects in other populations, for example those at risk for T2DM or patients diagnosed with T2DM. In addition, the large gap between metabolic improvements upon NR observed in mice and the absence of effects in humans may originate from the fact that mice studies applied a much longer supplementation period (up to 15 weeks) – especially when this supplementation period is considered relative to the lifespan – compared to short-term human clinical trials (maximum of 12 weeks). Another possibility might be a lower tissue bioavailability of oral NR supplementation in humans compared to mice. More research should be done to unravel the exact pharmacokinetic properties of NR in humans and the possible differences between mice and humans. A recently identified new NAD⁺-precursor, a reduced form of NR called NRH, acts via a slightly different metabolic pathway and has been shown to be more potent in increasing NAD⁺ levels in mice tissue compared to NR (92). For NRH, the next step would be to evaluate safety and tolerability of NRH in humans and eventually investigate whether NRH might have the hypothesized effects on human metabolic health which has not been shown so far upon NR.

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APPENDICES

Valorization

Summary

Samenvatting

Dankwoord

List of publications

About the author

VALORIZATION

SOCIAL RELEVANCE

Obesity is a worldwide increasing problem, warranting for new prevention and treatment strategies to reduce the risk on developing chronic diseases related to excess body weight. According to the 2016 report of the World Health Organization (WHO) the prevalence of obesity is still increasing worldwide, with 39% of adults aged 18 years and older being overweight (> 1.9 billion adults) and 13% being obese (>600 million adults) (1). It is especially alarming that 41 million children under the age of 5, and 340 million children and adolescents aged 5-19 were overweight and obese in 2016 (1). Overweight and obesity are major risk factors for the development of type 2 diabetes mellitus (T2DM). Just like obesity, the prevalence of T2DM has been steadily increasing over the past few decades. T2DM is associated with various comorbidities, including cardiovascular diseases and non-alcoholic fatty liver disease as well as an increased mortality risk. The WHO underscores in their global report on diabetes the enormous scale of the diabetes problem, and also the potential to reverse current trends (2). It has also been pointed out by the WHO that obesity is preventable (1). Furthermore, T2DM is one of the four priority noncommunicable diseases (NCDs) targeted by world leaders in the 2011 Political Declaration on the Prevention and Control of NCDs (2). These reports and declarations highlight the importance of the prevention and treatment of obesity and T2DM worldwide.

The fundamental causes of obesity and T2DM include a high caloric diet and insufficient physical activity. Effective tools are available to prevent obesity and T2DM and to improve disease management to reduce the risk of complications. Promoting physical activity is considered one of the most potent strategies to prevent and treat T2DM. World leaders agreed that one of the global targets for the prevention of T2DM (and other NCDs) to be attained by 2025 is a 10% relative reduction in the prevalence of insufficient physical activity (2). Other effective strategies for the prevention and treatment of obesity and T2DM include a healthy diet low in calories. Existing strategies that stimulate physical activity and promote a healthy diet are effective in reducing body weight and improving glucose homeostasis in patients with obesity and T2DM. However, these strategies seem to be challenging to adhere to on the long term for the people at risk. Therefore, it is important to investigate other strategies to prevent and treat obesity and T2DM. The present thesis describes three novel approaches that could lead to novel prevention and treatment strategies for people with obesity and obesity-related comorbidities such as T2DM and cardiovascular diseases.

Supplementation with the NAD⁺ precursor nicotinamide riboside has been investigated in this thesis as the first novel approach. Nicotinamide riboside can be easily added to the normal diet, which would enhance the compliance for the general public in contrast to low compliance rates for exercise training or a rigorous diet. Although it has been shown that nicotinamide riboside had many metabolic benefits including improvements in insulin sensitivity in animal models, these effects could so far not be translated to humans, as also shown in this thesis. However, since a relative short intervention period was applied in this thesis, future research should investigate whether long term supplementation with nicotinamide riboside could have metabolic benefits in humans to prevent or treat obesity and T2DM. The second novel approach that was investigated in this thesis was non-shivering cold acclimation. From this thesis we conclude that non-shivering cold acclimation for ten days at 16-17 °C does not have major beneficial health effects in humans with T2DM. However, it could be suggested that mild shivering induced by cold environmental temperatures, either indoor or outdoor, could be a novel way to improve metabolic health for the general public. More research is needed to quantify the amount and intensity of shivering that is needed to contribute to the prevention and treatment of obesity and T2DM, and the practical applicability in daily life. As a third novel approach we investigated whether replacing sitting time with low-intensity physical activities would have the same metabolic benefits compared to replacing sitting time with a single moderate-to-vigorous exercise bout per day. In this thesis we demonstrated that low-intensity physical activities such as standing and walking are similar effective in improving insulin sensitivity compared to a single moderate-to-vigorous exercise bout per day. Taken together, investigating novel approaches that have beneficial health effects and that can be implemented in daily life are of great social relevance to prevent and treat obesity, T2DM and other metabolic diseases.

ECONOMIC RELEVANCE

Obesity, T2DM and related comorbidities are a major economic burden for national health care systems, as well as for the patients and their families. In the Netherlands, total health care costs for T2DM (including comorbidities) were 1.6 billion euro's in 2017 (3). Preventing obesity and T2DM via inexpensive and easy to access strategies such as the ones presented in this thesis can contribute to a reduction in global economic burden. Increasing physical activity by standing and walking is not only freely accessible and inexpensive for the general public, it does also contribute to a reduction in transportation use and costs. Food supplements, such as nicotinamide riboside, can be an inexpensive and easy addition to a healthy diet. Furthermore, lowering indoor temperatures and thereby challenging the body to deal with cooler environments, cannot only could improve human health but can also could reduce

heating costs of homes, offices and buildings. Above all, a reduction in transportation and heating contributes to less emission of greenhouse gasses. Scientific evidence exists that warming of the climate system is unequivocal and that greenhouse gasses emitted by human activities are the primary driver (4). The Paris Agreement signed in 2016 by 195 countries, states that by 2050 the emission of greenhouse gasses has to be reduced by 95% in comparison to the level in 1990 (5, 6). As obesity and T2DM is an increasing problem and at the same time climate change is an urgent matter, the investigation of novel health promoting strategies to prevent and treat obesity and T2DM that are at the same time climate-friendly are of great economic relevance.

ACTIVITIES AND PRODUCTS

All studies described within this thesis were executed at the Department of Nutrition and Movement Sciences at Maastricht University within the Diabetes and Metabolism Research Group. The Diabetes and Metabolism Research Group investigates the mechanisms underlying the pathogenesis of T2DM and related metabolic diseases by performing translational research. The studies presented in this thesis were performed in close collaboration with the Department of Radiology and Nuclear Medicine of Maastricht University Medical Centre. This collaboration between scientists and the clinic allowed us to use non-invasive techniques like MR and a PET-CT scanning. Furthermore, research presented in this thesis is part of a large consortium (CVON ENERGISE 2014-02) between Amsterdam Medical Center, Leiden University Medical Center, Wageningen University and Maastricht University. Being part of such a consortium enhances collaborations between the different centers and thereby improves translational research.

The results presented in this thesis are or will be published in original scientific articles in international well-recognized peer-reviewed journals. Importantly, articles will be available online and can be assessed by scientists or other interested people worldwide. Furthermore, results from the studies described in this thesis are communicated and presented to the scientific community on (inter)national conferences and symposia via oral presentations and posters. Via this way, research knowledge was spread within the scientific community to facilitate new research ideas and hypotheses and to promote future research.

INNOVATION

The study results presented in this thesis are obtained in humans by state-of-the-art methods and techniques. When human clinical trials are performed, it is of great importance that reliable results are obtained in the most non-invasive way possible. In this thesis, we applied relatively new MRS techniques developed by our research

group to examine among others lipid accumulation in the muscle and muscle acetylcarnitine dynamics in a non-invasive way. In the past, these outcomes could only be studied and quantified with the use of tissue biopsies. Furthermore, we used stable isotope tracers during the hyperinsulinemic-euglycemic clamps and during the assessment of brown adipose tissue activity in combination with PET scanning. We also used a metabolic chamber to assess sleeping metabolic rate upon NR supplementation and to control the indoor environment with a stable temperature during the cold acclimation study period. Collaborations within the ENERGISE consortium allowed us to use additional technologies, such as metabolomics, to obtain more results from the valuable muscle tissue samples. The combination of these advanced techniques can only be performed in a few research departments worldwide and leads to innovative translational research data.

Furthermore, many research has been performed on classic intervention strategies such as exercise training (endurance training, resistance training, high intensity interval training or a combination) and reducing caloric intake with various diets (such as low carbohydrate diet, high protein diet, low fat diet, low glycemic load diet, Mediterranean diet, paleo diet, intermittent fasting or a combination). These intervention strategies share a common goal: increasing energy expenditure and reducing energy intake resulting in a low cellular energy status. A low cellular energy status activates metabolic pathways that lead to metabolic adaptations which are favorable for human health, such as an increase in insulin sensitivity. The research presented in this thesis describes the investigation of novel approaches to improve insulin sensitivity by targeting the same metabolic pathways as are activated by exercise training and caloric restriction, but then with novel strategies: supplementation with the food supplement nicotinamide riboside, non-shivering cold acclimation, and replacing sitting time by low-intensity physical activities.

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SUMMARY

The worldwide prevalence of obesity and type 2 diabetes mellitus (T2DM) has dramatically increased over the past decades, and this development is also seen in the Dutch population. Obesity can lead to reduced insulin sensitivity and mitochondrial dysfunction, which are key characteristics of metabolic diseases such as T2DM. The fundamental cause of the high prevalence of obesity and T2DM is an unhealthy lifestyle characterized by a high caloric diet and insufficient physical activity. Exercise training and dietary caloric restriction are the two most commonly studied and most powerful lifestyle interventions to counteract obesity and T2DM. These intervention strategies share a common goal: inducing a low cellular energy status by increasing energy expenditure or reducing energy intake respectively. A low cellular energy status activates metabolic pathways that induce metabolic adaptations which are favorable for human health, such as an increase in insulin sensitivity and mitochondrial function. However, these strategies seem to be challenging to adhere to on the long term for people with obesity and T2DM. Therefore, it is important to investigate alternative strategies to prevent and treat obesity and T2DM. The research presented in this thesis describes the investigation of novel approaches to improve human insulin sensitivity and muscle mitochondrial function by targeting the same metabolic pathways as are activated by exercise training and caloric restriction, but with novel strategies. For this, three human intervention studies have been performed.

The first novel approach we investigated in this thesis was dietary supplementation with the NAD⁺-precursor nicotinamide riboside (NR). NAD⁺ is a critical signaling molecule of cellular energy status and is an essential substrate for the NAD⁺-dependent enzyme sirtuin 1. Sirtuin 1 is a metabolic regulator of oxidative energy metabolism and is activated in situations of a low cellular energy status. Since, NAD⁺ levels are decreased in the obese population, replenishment of NAD⁺ levels was hypothesized to be a new strategy to improve metabolic health. Therefore, in **chapter 2 and 3**, the effect of six weeks 1000 mg/day NR supplementation in healthy overweight and obese humans has been investigated on a wide range of metabolic health outcomes in a randomized placebo-controlled double-blinded cross-over intervention study. In **chapter 2** we show that NR supplementation increased skeletal muscle NAD⁺ metabolite levels NAAD and MeNAM, without affecting NAD⁺ content itself. This suggests enhanced NAD⁺ turnover in skeletal muscle. Furthermore, NR induced changes in skeletal muscle acetylcarnitine metabolism, increased sleeping metabolic rate, and induced small improvements in body composition. However,

besides these modest effects, no other metabolic health effects were observed regarding insulin sensitivity, muscle mitochondrial function, ectopic lipid accumulation, whole body substrate oxidation, blood pressure, cardiac energy status and plasma markers of inflammation and energy metabolism. In **chapter 3**, the effects of NR supplementation on brown adipose tissue (BAT) activity were evaluated. We show that NR supplementation in HFD-fed mice did enhance BAT activity. In addition, *in vitro* adipocytes derived from human BAT treated with NR did increase mitochondrial uncoupling. However, these promising preclinical results were not translated in humans, as the results from the human intervention study indicate that NR supplementation did not affect human BAT activity or cold induced thermogenesis.

The second novel approach we investigated in this thesis was non-shivering cold acclimation. In **chapter 4** we investigated whether previously reported beneficial effects of ten days mild cold acclimation on peripheral insulin sensitivity were sustained for a longer period of time upon cessation of the cold exposure. Furthermore, we investigated whether these effects were accompanied by improvements in postprandial glucose and lipid metabolism and markers of cardiovascular health. For this, a human intervention study was performed in patients with T2DM who were exposed to mild cold (16-17 °C) for ten consecutive days. During the cold acclimation, we aimed to prevent shivering and targeted to investigate the effects of non-shivering thermogenesis. Using this approach, we could not reproduce the improvements in peripheral insulin sensitivity upon mild cold exposure that has previously been reported. Hence, also no improvements in the other outcomes were observed. To investigate the underlying reason for this discrepancy in results regarding insulin sensitivity, we performed additional analyses in skeletal muscle biopsies from the previous cold acclimation study and our current study. In samples from the previous cold acclimation study it was observed that the expression of gene-sets related to muscle contraction was induced along with increased GLUT4 translocation to the cell membrane. In the current study, specifically designed to prevent shivering, no such changes were observed. Jointly, this may indicate that shivering may have occurred in the previous study, which may have resulted in the induction of contraction related gene sets and more GLUT4 in the muscle cell membrane upon cold acclimation.

In recent years, both epidemiological and intervention studies have shown that replacement of sedentary time by light-intensity physical activities (such as standing and walking), as well as moderate-to-vigorous physical activities (such as exercise training) induced improvements in glucose homeostasis and markers of insulin

sensitivity, suggesting improved metabolic health. Thus far, it is not known, however, if these improvements act via similar underlying pathways. **Chapter 5** describes the third novel approach we investigated in this thesis. A randomized cross-over intervention study was performed in which healthy overweight and obese women underwent a four-day sit regime, exercise regime and sit less regime followed by metabolic assessments on the fifth day. We show that replacing sedentary time by several hours of standing and walking (sit less regime) was similarly effective in improving human peripheral insulin sensitivity compared to a single moderate-to-vigorous exercise bout per day (exercise regime). This improvement in peripheral insulin sensitivity could not be explained by an increase in muscle mitochondrial function. Furthermore, hepatic insulin sensitivity and intrahepatic lipid accumulation were not affected by the four-day activity regimes.

Overall, the human intervention studies described in this thesis were designed to investigate novel approaches to improve human metabolic health with the main aim to improve human insulin sensitivity and muscle mitochondrial function. Taken together, NR supplementation and cold acclimation without shivering did not improve human insulin sensitivity or other important markers of metabolic health. It could be suggested that mild shivering upon cold exposure might be an effective strategy to increase peripheral insulin sensitivity. Furthermore, short term replacement of sitting time by standing and walking or by exercise is similarly effective in promoting human insulin sensitivity while these effects were not mediated by improved muscle mitochondrial function.

SAMENVATTING

De prevalentie van obesitas en type 2 diabetes mellitus (T2DM) is wereldwijd sterk toegenomen in de afgelopen decennia, en deze ontwikkeling is ook zichtbaar in Nederland. Obesitas leidt tot een verminderde insuline gevoeligheid en mitochondriële functie, welke beide belangrijke kenmerken zijn van metabole ziekten zoals T2DM. Een ongezonde leefstijl bestaande uit een hoge calorie inname en weinig fysieke activiteit is een belangrijke oorzaak van de hoge prevalentie van obesitas en T2DM. De meest bestudeerde en effectieve leefstijlinterventies om obesitas en T2DM tegen te gaan, zijn sporten en een dieet laag in calorieën. Deze leefstijlinterventies zorgen beiden voor een negatieve energiebalans. Een negatieve energiebalans activeert specifieke metabole routes die ervoor zorgen dat onder andere insuline gevoeligheid en mitochondriële functie verbeteren. Dit is gunstig voor de metabole gezondheid. Echter, meer sporten en een lagere calorie inname zijn moeilijk vol te houden op de lange termijn voor mensen met obesitas en T2DM. Daarom is het belangrijk dat er onderzoek wordt gedaan naar alternatieve strategieën om obesitas en T2DM te behandelen en te voorkomen. Dit proefschrift beschrijft onderzoek naar nieuwe strategieën om insuline gevoeligheid en mitochondriële functie te verbeteren door middel van het activeren van dezelfde metabole routes die ook worden geactiveerd door sporten en een lagere calorie inname. Zodoende zijn er drie humane interventie studies uitgevoerd en beschreven in dit proefschrift.

De eerste nieuwe interventie strategie die is onderzocht in dit proefschrift is het voedingssupplement nicotinamide riboside (NR). NR wordt in het lichaam omgezet naar NAD⁺. NAD⁺ is een molecuul dat in concentratie toeneemt bij een lage energie status in cellen, zoals bij een negatieve energiebalans. Daarnaast is NAD⁺ een essentieel substraat voor het enzym sirtuin 1. Sirtuin 1 speelt een belangrijke rol in het regelen van het energie metabolisme. Concentraties van NAD⁺ zijn verlaagd in mensen met obesitas. Er wordt dus verwacht dat een verhoging van NAD⁺ concentraties in deze doelgroep zal leiden tot een verbeterde metabole gezondheid. In **hoofdstuk 2 en 3** is een studie beschreven waarin de effecten van zes weken 1000 mg/dag suppletie met NR in gezonde mensen met overgewicht en obesitas is onderzocht op verschillende metabole uitkomstmaten. De studie was gerandomiseerd, placebo-gecontroleerd, dubbelblind en had een cross-over onderzoeksopzet. In **hoofdstuk 2** laten we zien dat suppletie met NR de concentratie van NAD⁺-gerelateerde metabolieten (NAAD en MeNAM) verhoogd in de spieren. De concentratie NAD⁺ zelf veranderde niet. Dit suggereert dat er een verhoogde NAD⁺ turnover was in de spieren. Daarnaast zorgde NR-suppletie voor een verandering in

het acetylcarnitine metabolisme van de spier, een verhoogd energiegebruik tijdens de slaap en een kleine verbetering in de lichaamssamenstelling. Ondanks deze resultaten had NR-suppletie geen effect op de andere metabole uitkomstmaten: insuline gevoeligheid, spier mitochondriële functie, vetopslag in de spier en lever, substraat verbranding, bloeddruk, energie status van het hart en plasma markers voor inflammatie en het energie metabolisme. In **hoofdstuk 3** worden de effecten van NR-suppletie op bruin vet activiteit beschreven. We laten zien dat in muizen NR-suppletie het bruin vet activeerde. Daarnaast laten we zien dat humane bruin vetcellen een verbeterde mitochondriële ontkoppeling hadden na toevoeging van NR *in vitro*. Desondanks zagen we deze resultaten niet terug in de humane interventie studie. Suppletie met NR in mensen met overgewicht en obesitas had namelijk geen effect op de bruin vet activiteit of op koude geïnduceerde thermogenese.

De tweede nieuwe interventie strategie die is onderzocht in dit proefschrift is koude acclimatisatie zonder rillen. In eerder onderzoek is aangetoond dat 10 dagen blootstelling aan milde kou de insuline gevoeligheid verbeterd. In **hoofdstuk 4** hebben we onderzocht of deze verbetering in insuline gevoeligheid daarnaast ook langer aanwezig bleef na het stoppen van de blootstelling aan de kou. Daarnaast hebben we onderzocht of deze verbetering in insuline gevoeligheid gepaard ging met een verbetering in het postprandiale glucose- en vetmetabolisme en in een verbetering in markers voor de cardiovasculaire gezondheid. Om dit te onderzoeken hebben we een humane interventie studie uitgevoerd waarin patiënten met T2DM werden blootgesteld aan milde kou (16-17 °C) voor 10 opeenvolgende dagen. Omdat we het effect van koude acclimatisatie zonder rillen wilden onderzoeken, hebben we ervoor gezorgd dat de proefpersonen zo min mogelijk rilden tijdens de koude blootstelling. Tegen onze verwachting in, zagen we geen effect van de koude acclimatisatie op de insuline gevoeligheid op de korte termijn, zoals in een eerdere studie wel was aangetoond. Vervolgens zagen we dus ook geen effect van koude acclimatisatie op insuline gevoeligheid op de langere termijn en ook niet op de andere metabole uitkomstmaten. Om te onderzoeken waarom wij de effecten van koude acclimatisatie op insuline gevoeligheid niet konden reproduceren, hebben we extra analyses gedaan in de spierbiopten uit de vorige en de huidige studie. Uit de analyses van de spierbiopten uit de vorige studie bleek dat de expressie van genen die gerelateerd zijn aan spiercontracties verhoogd waren en tegelijk ook de translocatie van GLUT4 naar de celmembraan verhoogd was. In de analyses van de spierbiopten uit de huidige studie waren deze effecten niet zichtbaar. Deze resultaten duiden erop dat in de vorige studie wellicht enige vorm van rillen aanwezig was tijdens de koude blootstelling, en in huidige studie niet. Spiercontractie en GLUT4 translocatie in de

vorige studie kan een verklaring zijn voor de gerapporteerde verbetering in insuline gevoeligheid.

De afgelopen jaren hebben epidemiologische en interventie studies aangetoond dat het vervangen van zittend gedrag door lichte fysieke activiteiten (zoals staan en wandelen), alsook door matig-tot-zware fysieke activiteiten (zoals sporten) het glucosemetabolisme kunnen verbeteren en waarschijnlijk ook insuline gevoeligheid en andere factoren gerelateerd aan de metabole gezondheid. Tot dusver is het nog onduidelijk of deze verbeteringen in het glucosemetabolisme bij de verschillende vormen van fysieke activiteit via dezelfde onderliggende metabole routes plaatsvinden. In **hoofdstuk 5** zijn de resultaten beschreven van de derde nieuwe interventie strategie die is onderzocht in dit proefschrift. Een humane interventie studie is uitgevoerd waarbij gezonde vrouwen met overgewicht en obesitas in een gerandomiseerde volgorde drie verschillende activiteitenregimes hebben gevolgd: een zit regime, een sport regime en een minder-zitten regime. Het sport regime bestond uit 1 uur sporten per dag met een matig-tot-zware intensiteit. Het minder-zitten regime bestond uit 4 uur staan en 3 uur wandelen per dag. De rest van tijd in het zit, sport en minder-zitten regime moest voornamelijk zittend worden doorgebracht. Elk activiteitenregime duurde 4 dagen en op de 5^e dag werden verschillende metingen verricht. We laten zien dat het vervangen van zittend gedrag door staan en wandelen (minder-zitten regime) net zo effectief was in het verbeteren van de spier insuline gevoeligheid als 1 uur sporten per dag (sport regime). Deze verbetering van de spier insuline gevoeligheid kon niet worden verklaard door een toename in spier mitochondriële functie. Verder lieten de resultaten zien dat de lever insuline gevoeligheid en de vetopslag in de lever niet veranderden na een activiteitenregime van 4 dagen.

Samenvattend, in dit proefschrift zijn drie humane interventie studies beschreven die waren ontworpen om nieuwe strategieën te onderzoeken om de humane metabole gezondheid te verbeteren met als hoofddoel het verbeteren van insuline gevoeligheid en spier mitochondriële functie. Er kan geconcludeerd worden dat NR-suppletie en koude acclimatisatie zonder rillen de insuline gevoeligheid en andere belangrijke parameters van metabole gezondheid niet kunnen verbeteren. Het wordt gesuggereerd dat een milde vorm van rillen tijdens koude acclimatisatie een manier is om insuline gevoeligheid te verbeteren. Tenslotte, 4 dagen vervanging van zittend gedrag door staan en wandelen is net zo effectief in het verbeteren van de spier insuline gevoeligheid als 1 uur sporten per dag en deze verbetering kan niet worden verklaard door een verbeterde spier mitochondriële functie.

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LIST OF PUBLICATIONS

Remie CME, Roumans KHM, Moonen MPB, Connell NJ, Havekes B, Mevenkamp J, *et al.* Nicotinamide riboside supplementation alters body composition and skeletal muscle acetylcarnitine concentrations in healthy obese humans. *Am J Clin Nutr.* 2020 Apr 22; nqaa072. Online ahead of print.

Roumans KHM, Lindeboom L, Veeraiah P, **Remie CME**, Phielix E, Havekes B, *et al.* Hepatic saturated fatty acid fraction is associated with de novo lipogenesis and hepatic insulin resistance. *Nat Commun.* 2020 Apr 20;11(1):1891.

Remie CME*, Moonen MPB*, Roumans KHM, Havekes B, Gemmink A, Nascimento EBM, *et al.* Non-shivering cold acclimation does not improve postprandial glucose and lipid metabolism or insulin sensitivity in patients with type 2 diabetes. *In preparation.*

* These authors contributed equally

Remie CME, Bilet L, Duvivier BMFM, Bergman M, Roumans KHM, Huysmans SMD, *et al.* Replacing sedentary time with standing and walking is similar effective in enhancing insulin sensitivity as moderate-to-vigorous exercise. *In preparation.*

Nascimento EBM*, Moonen MPB*, **Remie CME**, Gariani K, Jörgensen JA, Schaart G, *et al.* Increasing NAD⁺ levels through nicotinamide riboside activates in vivo brown adipose tissue in mice, but not in humans. *Submitted.*

* These authors contributed equally

ABOUT THE AUTHOR

Carlijn Remie was born on March 12th, 1991 in Sittard, the Netherlands. After finishing secondary education at Trevianum Sittard in 2009, she started the bachelor Nutrition and Dietetics at the HAN University of Applied Sciences in Nijmegen. After successfully completing her Bachelor's degree in 2013, Carlijn continued her education with the Master Nutrition and Health at the Wageningen University. She performed her Master thesis at the Department of Human and Animal Physiology of Wageningen University investigating the effects of carnitine supplementation on acute response to hypoxia in humans. For her Master internship she visited the University of Copenhagen, department of Biomedical Sciences, for five months. During her internship in Copenhagen, she investigated the effects of intermittent fasting on intramyocellular triglyceride content of human skeletal muscle and assisted in conducting a human clinical trial. In 2015, Carlijn received her Master's degree and subsequently started her PhD at the department of Nutrition and Movement Sciences at Maastricht University under the supervision of Prof. dr. Patrick Schrauwen, Dr. Esther Phielix and Prof. dr. Matthijs Hesselink. The research conducted during this period, as described in this PhD thesis, was primarily focused on the investigation of novel approaches to improve human insulin sensitivity and muscle mitochondrial function.