A metabolically healthy lifestyle

A metabolically healthy lifestyle: A matter of timing?

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A metabolically healthy lifestyle: a matter of timing?

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CONTENT

CHAPTER 1

General introduction and outline

Life on earth has evolved mechanisms to efficiently cope with the predictable changes in the environment resulting from the rotation of the earth around its axis and around the sun. It has been hypothesized that these timed mechanisms have initially evolved as a response to the UV-irradiation from the sun that causes damage to the DNA, which needs to be repaired during the dark period, i.e. at night time (1). Indeed, humans have also developed a rhythmic system that allows to optimally respond to predictable changes in external environmental factors (e.g. temperature and light exposure) as well as to predictable changes in internal physiological processes due to behaviour (e.g. food intake and physical activity). To this end, virtually all cells and tissues exhibit an internal, circadian (circa is about, diem is day) rhythm (2, 3). In turn, this internal rhythm can be altered by stimuli that indicate the time-of-day (*Zeitgebers*, i.e. food intake, exercise, light exposure, temperature) resulting in a rhythm that is attuned to day and night (day-night rhythm). The main driver of internal rhythms is located in the suprachiasmatic nucleus (SCN), and uses the light input signal from the retina to synchronize circadian rhythms to the exact 24 h geophysical day. The ability of circadian rhythms to alter in response to *Zeitgebers* enables humans to still respond effectively when there is a shift in environmental rhythms, such as the annual shift in seasons or when travelling through time zones. However, recent advances in circadian research show that too frequently challenging the internal day-night rhythm, as with night shift work, increases the risk for metabolic diseases, including type 2 diabetes (4-6).

In our current society, environmental- and behavioural rhythms are no longer predictable as modern technology allows for food intake and activity at any time-of-day. Indeed, most people in Western society appear to spread their daily food intake over at least 14 h (7, 8), spending most of their day in a fed state. Additionally, in modern 24 h society there is a demand for night shift workers (9, 10), who, on a regular basis, shift their day-night rhythm by eating and being active at times that they would normally sleep. In parallel to the technological advances that have preceded this 24 h society, there has been a widespread progressive increase in the prevalence of type 2

diabetes (11). It is interesting to hypothesize that this increase in type 2 diabetes can be partially explained by the frequent disturbances in the day-night rhythm and the current 24 h culture we live in. This notion is supported by studies that show that simulating a night shift in an experimental setting (circadian misalignment) results in deterioration of insulin sensitivity in young, healthy volunteers [12-14]. Moreover, already a slight shift in the day-night rhythm induced by changes in bedtime and time of waking up due to societal pressures, social jetlag, has been related to impaired metabolic health [15, 16]. Thus, eating and being active at times that the body is not prepared for it appears to harm metabolic health. Conversely, males at risk for type 2 diabetes have been found to exhibit a dampened day-night rhythm in substrate oxidation and mitochondrial function when compared to the rhythms found in healthy lean males (17, 18). These findings call for timed interventions that aim to restore the natural day-night rhythm and thereby improve metabolic health.

In this thesis, it is examined if re-aligning the timing of exercise and food intake with the natural day-night rhythm and thereby restoring the fasting-feeding cycle, has the potential to improve metabolic health.

Thesis outline

The aim of this thesis is to investigate opportunities to improve metabolic health by reinforcing the natural day-night rhythm via timed lifestyle interventions. **Chapter 2** entails a review wherein the working mechanisms of the circadian rhythms are discussed and which evaluates the potential of a range of timed lifestyle interventions in improving metabolic health.

In addition to circadian disruption, a disordered sleep is also related to impaired metabolic health (19-22). An intimate relationship between the circadian rhythm and sleep exists, as sleep is dictated by both the homeostatic sleep pressure, that increases as the day progresses, and the circadian rhythm (23). Thus, it may be possible that

reinforcing the circadian rhythm also results in an improved sleep quality. In this perspective, altering the timing of intense physical activity seems promising, as exercise is an established lifestyle intervention to improve sleep (24), and, additionally, acts as a time cue for circadian rhythms. Therefore, **Chapter 3** is dedicated to investigate if a relationship between the time-of-day of moderate-to-vigorous physical activity and sleep quality and duration exists.

Although physical activity is an important *Zeitgeber* in the composition of circadian rhythms, the rhythm in food intake and fasting is believed to be the dominant *Zeitgeber* for metabolic tissues, such as the liver and muscle (25). For this reason, Chapters 4 – 7 are devoted to revealing the metabolic effects of timing of food intake and fasting in different experimental protocols. **Chapter 4** involves a randomized controlled cross-over trial in which the metabolic effects of an acute 16 h versus 9.5 h fast are being assessed in both overweight/obese adults with non-alcoholic fatty liver (NAFL) disease, and, as a control, in healthy lean adults. In this study, we were specifically interested in fluctuations of hepatic glycogen, since the liver plays an important role in the supply of glucose during a prolonged fast by means of glycogenolysis. Moreover, exhaustion of glycogen stores triggers (molecular) fasting mechanisms and consequently results in an increased fat oxidation (26). Thus, for this proof-of-concept study, we hypothesized that prolonging the daily fasting period, thereby matching the natural day-night rhythm, resulted in an increased hepatic glycogen turnover and -nocturnal fat oxidation. These effects could be especially beneficial for people with NAFL, since they have a high level of hepatic fat accumulation which may be reduced when fat oxidation increases.

In **Chapter 5**, we assess if repeatedly prolonging the overnight fast for 3 weeks is able to decrease fasting hepatic glycogen stores and improve insulin sensitivity in adults with type 2 diabetes. We hypothesized that utilization of hepatic glycogen stores during the night would result in an increased need to replenish these stores with the first morning meal and, by repeatedly doing so, would result in an improved insulin

sensitivity. In this randomized cross-over trial, volunteers were instructed to limit their habitual food intake to a 10 h diurnal time frame (time restricted eating, TRE). As a control, participants were instructed to spread their habitual food intake over at least 14 hours per day, without constrains regarding the eating time window.

Chapter 6 takes a different approach to restoring the fasting–feeding cycle by pharmacologically inducing a more pronounced fasting state during the night using the sodium-glucose cotransporter 2 inhibitor (SGTL2i) dapagliflozin. Dapagliflozin is a drug that is used with type 2 diabetes and inhibits the resorption of glucose at the renal tubule and thereby causes a higher urinary glucose outflux (glucosuria). Here, we hypothesized that two weeks of dapagliflozin (10 mg/day) would result in higher nocturnal hepatic glycogen utilization and an improved mitochondrial function as compared to the placebo. To investigate this, a randomized controlled trial was conducted in adults with insulin resistance.

In **Chapter 7** we explore the role of food intake as a *Zeitgeber* for the day-night rhythm in substrate oxidation. For this purpose data has been used from a fasting study whereby healthy, lean male volunteers were fasted for 60 h. During this time, participants remained in a respiration chamber to examine substrate utilization and energy expenditure using whole-room indirect calorimetry.

Finally, **Chapter 8** merges the major findings of the various studies discussed in this thesis and aims to gain a better understanding of the potential of timed interventions to improve metabolic health.

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Chapter 2

The importance of 24h metabolism in obesity-related metabolic disorders: opportunities for timed interventions

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Abstract

Various metabolic processes in the body oscillate throughout the natural day, driven by a biological clock. Circadian rhythms are also influenced by time cues from the environment (light exposure) and behaviour (eating and exercise). Recent evidence from diurnal- and circadian rhythm studies indicates rhythmicity in various circulating metabolites, insulin secretion and -sensitivity and energy expenditure in metabolically healthy adults. These rhythms have been shown to be disturbed in adults with obesity-related metabolic disturbances. Moreover, eating and being (in)active at a time that the body is not prepared for it, as in night shift work, is related to poor metabolic outcomes. These findings indicate the relevance of 24h metabolism in obesity-related metabolic alterations and have also led to novel strategies, such as timing of food intake and exercise, to reinforce the circadian rhythm and thereby improving metabolic health. This review aims to deepen the understanding of the influence of the circadian system on metabolic processes and obesity-related metabolic disturbances and to discuss novel time-based strategies that may be helpful in combating metabolic disease.

Introduction

Already 77.000 years ago, the Maya's were able to keep track of time using naturally occurring, predictable events such as the rising and setting of the sun, and adapted their behavioural rhythms accordingly (1). As a result, the Maya's ate and were active during the day when there was sunlight, whereas they slept during the night. Indeed, the human body has an internal biological clock that is tuned by recurring events such as light exposure, food intake and exercise. Fascinatingly, even without these external cues, clocks in various tissues of the human body appear to generate a rhythm. This internal rhythm takes approximately 24-hrs to complete and is referred to as the circadian rhythm ("circa" *lat* about, "diem" *lat* day) (2, 3). Environmental time cues (or *Zeitgebers*), i.e. light exposure, entrain the internal clock and synchronize the biological rhythm of numerous processes in the human body to the 24h geophysical day. Behavioural time cues can also act as *Zeitgebers* and further modulate internal biological rhythms. The biological clock has been hypothesized to anticipate predictable external cycles that occur during the day in order to generate an optimal homeostatic response (3, 4).

In current Western society, food intake and physical activity are often no longer attuned to the natural light-dark cycle, since technology makes it possible to eat and be active at any time of the day. As a result, misalignment of the behavioural rhythm with the internal circadian rhythm can occur, i.e. people may eat and be physically (in)active at times when the body is not optimally prepared for it. Indeed, it has been shown that most people spread their food intake over a 15-hrs time period which also includes evening hours (5). Furthermore, approximately one in five people of the European working population works in shifts, including work during the night when the body is prepared for sleep (6). In addition to shift work, people also experience circadian misalignment when travelling across different time zones (jetlag) or even by staying up late and sleeping in longer during the weekend as compared to weekdays (social jetlag) (7).

Epidemiological studies show that shift work is associated with body weight gain, impaired glucose tolerance and an increased risk for type 2 diabetes (T2D) (8, 9). In addition, social jetlag has been associated with impaired metabolic health, metabolic syndrome and T2D (10-12). Moreover, a 12-hours shift in day and night rhythm, imposed in an experimental setting, already led to a transient decrease in skeletal muscle insulin sensitivity and an increase in blood pressure and inflammatory markers of otherwise healthy adults (13, 14). Therefore, it is evident that disturbances in the circadian system are negatively linked to metabolic health.

This review aims to provide insight into the working mechanisms of the biological clock and its role in the pathogenesis of obesity-related metabolic disturbances in humans. Furthermore, opportunities to reinforce the circadian system thereby improving metabolic health, for example by adjusting the timing of lifestyle factors, will be discussed.

The working mechanism of the biological clock

In the human body, circadian rhythms are generated by a master pacemaker (or central clock) that is located in the suprachiasmatic nucleus (SCN) of the posterior hypothalamus and that works in close connection with auxiliary clocks located in peripheral tissues (figure 1) (15). The SCN uses environmental light to synchronize the body's approximate 24-hrs circadian rhythm with the actual 24h geophysical day (15). Light is sensed by a small number of retinal ganglion cells in the eyes that express the photo-pigment melanopsin. Subsequently, the light signal travels to the SCN via the retino-hypothalamic tract (16). The SCN entrains the rhythms of the peripheral tissues to the light-dark cycle via the autonomic nervous system, hormonal signals (including melatonin and cortisol), body temperature and by its influence on e.g. food intake behaviour (15, 17-19). Next to the central clock in the SCN, peripheral clocks are present in virtually all tissues of the body and, even when disconnected from the SCN, they exhibit their own, internal rhythms (2).

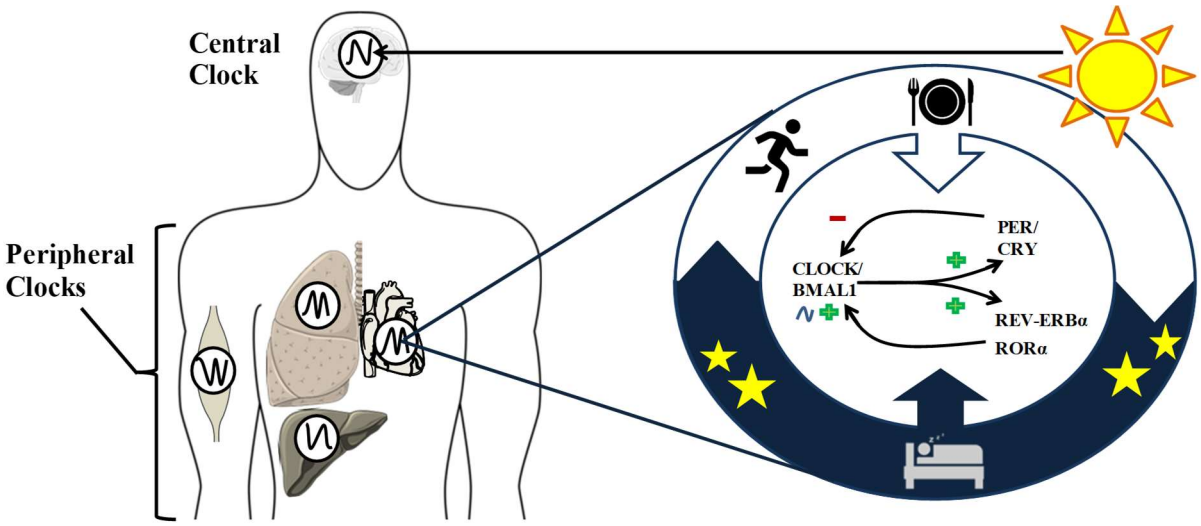

Figure 1. **Organization of circadian rhythms in the human body.** The master pacemaker is located in the SCN of the hypothalamus and its main time cue is light. In turn, intrinsic peripheral clocks in the body are present, which are aligned to the 24h environment by both the SCN and behavioural rhythms, including sleeping, activity and eating. The molecular driver for circadian rhythms is a transcriptional-translational feedback loop with a positive limp consisting of the BMAL1/CLOCK complex and a negative limp consisting of PER and CRY. An auxiliary feedback loop consisting of REV-ERBα and RORα adds to the robustness of the oscillatory mechanisms by driving the rhythmic BMAL1 expression.

On a molecular level, circadian rhythms are driven by transcriptional-translational feedback loops. The primary feedback loop comprises the transcription factors BMAL1 (Brain and Muscle ARNT-Like1) and CLOCK (Circadian Locomotor Output Cycles) that form a heterodimeric complex which drives the transcription of the repressor proteins PER (Period) and CRY (Cryptochrome) by activating the Per and Cry genes (20). In turn, PER and CRY form an inhibitory complex that, in sufficient concentrations, inhibits the activity of CLOCK-BMAL1. In turn, lower activity of CLOCK-BMAL1 results in a decreased PER and CRY expression. When PER and CRY protein levels are below a certain threshold, they stop inhibiting CLOCK-BMAL1 thus allowing a new cycle of PER

and CRY accumulation (figure 1) (20). A secondary feedback loop is formed by the expression of genes encoding REV-ERBα (nuclear receptor subfamily 1 group D) and RORα (RAR-related orphan receptor) which bind to the same element within the Clock and Bmal1 promotor regions and thus drive the rhythmic expression of Bmal1 (21, 22). In peripheral clocks, signals emanating from the behavioural rhythm, e.g. feeding/fasting, can also modulate the clock machinery by means of post-translational modification (22, 23). For example, AMP-activated protein kinase (AMPK), the cellular energy sensor, has been shown to modulate the clock mechanism by phosphorylation of PER and CRY (24, 25).

Taken together, the internal time keeping system of the body consists of both a central clock and peripheral clocks that are molecularly driven by transcriptional-translational feedback loops (figure 1). The end result of this molecular machinery is an overt rhythm in physiology and behaviour, which is further modulated by the interplay of the body clocks and environmental and behavioural signals. This timekeeping system enables the body to respond efficiently to predictable disturbances in homeostatic processes (3, 4).

Out of tune? Circadian rhythmicity in relation to metabolic perturbations

The influence of the circadian system in metabolism is visible through diurnal rhythms that have been found in various important metabolic parameters, including circulating metabolite levels, insulin secretion and –sensitivity as well as energy expenditure (26-31). Thus, over a 24h period, glucose levels in both healthy lean and overweight individuals have been reported to be highest in the morning (28, 31). In healthy lean males, fasting insulin was found lowest in the early morning compared to noon and midnight when assessed at four different time points on separate occasions with fasting time standardized before each measurement (32). However, other studies did

not find a clear difference between morning vs. afternoon levels of fasting insulin in lean and overweight adults (31, 33), not even when fasting time was standardized (31). The discrepancy in results might be explained by the time points at which fasting insulin levels were measured, since also insulin secretion has been shown to be rhythmic (26). Thus, using a 68-hrs hyperglycaemic clamp, healthy lean participants exhibited the highest insulin secretion during the day and the lowest during the night (26). In addition, meal tests or glucose/insulin tolerance tests performed at different times of day revealed that glucose tolerance of healthy individuals is highest in the morning and lowest in the afternoon and evening, and that this rhythm appears to be at least partially mediated by the rhythms of both insulin sensitivity and β-cell responsivity (27, 29, 33-40). Interestingly, when assessed in the evening, glucose tolerance in metabolically healthy individuals appeared to be metabolically equivalent to the pre-diabetic state (as measured in the morning) (39, 40).

Fasting levels of free fatty acids have also been reported to show rhythmicity and appeared to be lower in the morning than in the afternoon (33) and evening (27), even when the fasting time before measurements was standardized (27). Triglycerides, on the other hand, have been shown to rise during the day and fall during the night when lean male participants received three main meals during daytime and measurements were performed throughout the 24h day (30). Using lipidomics approaches, diurnal oscillations of lipid metabolites have also been shown in skeletal muscle tissue from healthy non-obese adults (41-43), which also persisted in primary muscle cell cultures (42). Besides oscillations observed in glucose homeostasis (also reviewed extensively elsewhere (29, 44) and various metabolites, studies also observed day-night rhythmicity in energy metabolism. Thus, the energetic cost of food intake, i.e. diet-induced thermogenesis (DIT), of a test meal identical in caloric value and macronutrient composition, was found to be highest in the morning and lowest during the night in lean and overweight individuals when two (45) or three (46) different clock times of meal intake were compared on separate occasions (45, 46). This response appeared to be irrespective of the fasting duration prior to the meal ingestion since

fasting time was standardized (45). Furthermore, we found that healthy lean men displayed a relatively higher glucose oxidation during the day and switched towards more fat oxidation during the night. Additionally, energy expenditure was highest during the late evening and lowest after midnight and this rhythm was partially paralleled by oscillations in oxidative capacity of the mitochondria, the energy producing organelles of the cells, which appeared to peak in the evening and had its trough just after noon (30). Together, these results show that both fat and glucose metabolism, as well as energy expenditure, are not static processes but rather oscillate over the course of a natural day.

Although these diurnal studies on 24h metabolic rhythms suggest the presence of circadian control, this cannot be concluded from the abovementioned studies since they do not eliminate the possibility that the findings are the result of (differences in) the environmental and/or the behavioural rhythm. Thus, the modulating effects of light exposure, food intake, and physical activity on the circadian system complicates the assessment of the contribution of the internal, independent circadian rhythm (see figure 1). To disentangle the behavioural and environmental rhythms from the internal circadian rhythm, specific study designs can be employed including the constant routine protocol, the inverted sleep-wake cycle protocol, the forced desynchrony protocol, and the misalignment protocol (see figure 2 for more information on these study designs). Thus, using a 26-hrs constant routine protocol (figure 2) in which healthy, lean female participants were seated in dim light and received hourly equicaloric drinks, endogenous circadian rhythms were found for glucose and triglyceride levels (both with higher levels during the night), but not for postprandial free fatty acids (47). A nocturnal peak in glucose levels, as well as in insulin secretion, was also found when healthy lean males, after a night of sleep, were being kept awake for 28 hrs and this period of wakefulness was followed by sleep during daytime (the inverted sleep-wake cycle protocol, figure 2) (48). This study, in which participants

were subjected to continuous glucose infusion while remaining fasted otherwise, also demonstrated that the sleep onset results in elevation of levels of plasma glucose and serum insulin and increases insulin secretion rate, irrespective of the time of day when sleep occurs (48). Furthermore, a 38 hr constant routine protocol found the peaks of insulin and glucose of healthy and overweight males to be around the usual time of awakening (49). In addition, endogenous circadian rhythms have also been found for energy expenditure. Thus, using a forced desynchrony protocol (figure 2) in which participants were subjected to a 28hrs behavioural rhythm, it was found that resting energy expenditure and substrate oxidation of healthy, lean to obese participants oscillates during the day with resting energy expenditure being highest during the day and lowest during the late night (50). Fasting carbohydrate oxidation proved to be highest in the biological morning whereas fasted fat oxidation peaked in the biological evening (50). Finally, a circadian misalignment protocol (figure 2) that shifted the day and night rhythm with 12-hrs showed that DIT of healthy lean and overweight adults was found to be highest in the morning and lowest in the evening in response to identical test meals (51). This circadian rhythmicity in DIT is in line with previous diurnal rhythm studies, indicating that the rhythm of DIT is not (entirely) driven by changes in behavioural rhythms (45, 46).

Taken together, various metabolic factors appear to exhibit specific day-night rhythmicity of which several are under endogenous circadian control, indicating that the time of day at least partially determines metabolic responses to homeostatic disturbances. The question then remains if obesity-related metabolic disturbances may be associated with disturbed circadian rhythmicity or inappropriate alignment of these (internal) circadian rhythms with external timing of behavioural/environmental rhythms.

Constant routine protocol: The participant remains awake and at rest, in a fixed posture and receives isocaloric meals at equal intervals under dim light conditions. The invariability of behavioural and environmental conditions allows to reveal the endogenous circadian rhythm.

Forced desynchrony protocol: Participants undergo behavioural cycles that are not 24 hrs (instead they are e.g. 28 or 20 hrs) and remain in dim light conditions. Since these cycles are outside the range of entrainment, the circadian system will express its internal circadian period. Behavioural rhythms are spread evenly across the circadian day-night cycle and this permits assessment of independent circadian rhythmicity.

Inverted sleep-wake cycle protocol: The participant remains awake for an extended time period and sleep is displaced to the daytime. In addition to the circadian rhythm, this protocol also allows to assess the effects of sleep irrespective of the time of day. Sometimes, participants are instructed to remain in a constant body posture, have minimal physical activity and are continuous supplied with nutrients via e.g. constant glucose infusion. This protocol takes advantage of the slow adaptation of the body to changes in environmental and behavioural rhythms and allows observation of independent circadian rhythms. The protocol is sometimes viewed as a form of forced desynchrony.

Misalignment protocol: The normal daily routine is shifted with a certain amount of hours often reflecting shift work and performed as part of a cross-over study that also entails an intervention arm reflecting the aligned situation. By using this protocol, the environmental and behavioural rhythms are uncoupled from the internal circadian rhythm, which enables the researcher to distinguish the behavioural effects, circadian phase effects and circadian misalignment effects.

Of note, in both the inverted sleep-wake and the constant routine protocol the forced wakefulness builds up sleep pressure which may affect the study variable of interest

Figure. 2 Various study protocols to examine circadian rhythmicity in humans. The different study protocols are used to reveal the independent circadian rhythm of metabolic processes by filtering out the modifying effects of the behavioural and environmental rhythm.

In this context, day-night rhythmicity has indeed been shown to be disturbed in people with impaired metabolic health. Cross-sectional studies revealed that the nocturnal peak in glucose level was two-fold higher in T2D patients in comparison with healthy matched controls during a prolonged 34-hours fast, a finding that could be replicated in some (28, 52), but not all (53), studies. In addition, both obese people with a normal fasting glucose as well as individuals with hyperglycaemia appear to lack rhythmicity of glucose tolerance since they do not display the typical higher glucose tolerance in the morning compared to the evening that is found in healthy lean individuals (38, 54). Furthermore, in obese patients with T2D a lack of rhythmicity in insulin secretion was observed during a 72-hours hyperglycaemic clamp (55), although insulin sensitivity showed a similar rhythm as reported for healthy lean adults (56). In addition, using continuous infusion of labelled glucose, it has been shown that the rates of endogenous glucose production were higher throughout the night in participants with T2D compared to the healthy controls, indicating an altered rhythm in hepatic glucose production in T2D (57, 58). These findings suggest altered rhythmicity of glucose metabolism in metabolically comprised individuals which could be indicative of a disturbed circadian system.

Indeed, there is some evidence to suggest that the putative differences in metabolic rhythmicity between people with obesity-related metabolic disturbances and healthy people relate to alterations in the central circadian system, although more research is needed on the nature of the altered rhythmicity of metabolism in metabolic diseases. In rodents, a lesion in the SCN resulted not only in the disappearance of rhythms in glucose uptake and insulin sensitivity, but also resulted in insulin resistance within 8 weeks after the SCN lesion was made, emphasizing the role of the central clock in regulating metabolic rhythmicity and insulin sensitivity (59, 60). Furthermore, mice studies showed that knockout of core clock genes, such as Bmal1, in peripheral metabolic tissues can lead to e.g. hypoglycaemia during fasting (61) or diminished

glucose-stimulated insulin secretion (62), which suggests that the peripheral clocks are also important in regulating metabolic processes. In humans, a recent study suggests that the SCN of people with T2D has a decreased number of arginine vasopressin and vasoactive intestinal polypeptide neuropeptides and astroglial cells as compared to healthy controls, which might underlie an altered metabolic circadian rhythmicity in T2D (63). Additionally, the circadian rhythm in cortisol levels, reflecting the direct output of the SCN, appears to be blunted in T2D patients as compared to individuals without T2D (64). Moreover, it is becoming increasingly evident that misalignment between the endogenous circadian rhythm and environmental and behavioural rhythms, i.e. eating and being active at times that the body is not prepared for it, is likely to hamper metabolic health. In this context, several meta-analyses showed that shift work, i.e. work performed during the nightly hours, is associated with a high BMI and an increased risk for impaired glucose tolerance and type 2 diabetes (T2D) (8, 65, 66). The probability to develop T2D appears to be higher when performing rotating night shift work as compared to rare shift work or permanent shift work, and a higher number of night shifts per month associates with higher probability of developing T2D (9). A large cohort study further found that the risk for T2D increased with 5% for every 5 years with a least three nights per month of performing rotating shift work (67). Shift-workers diagnosed with T2D also displayed higher Haemoglobin A1c levels, body mass index and daily caloric intake as compared to diabetes patients not involved in shift-work (68). Interestingly, cohort studies have shown that the typical shift in day-night rhythm that occurs during the weekend (social jetlag) already associates with a worsened glucose regulation and lower metabolic health and increases the risk on metabolic syndrome and T2D (11, 12, 69). More direct evidence for a causal relation between circadian misalignment and metabolic health comes from experimental studies that artificially shifted the normal day and night rhythm of healthy participants under strictly controlled conditions (see figure 2). Such circadian misalignment studies revealed that a disturbed circadian rhythm results in a decreased total daily energy expenditure but elevated sleeping metabolic rate, and increased levels of glucose,

insulin, ghrelin, free fatty acids, triacylglycerol and inflammatory markers as well as an increased 24-hrs systolic- and diastolic blood pressure (10, 13, 14, 70-73). Moreover, several independent studies reported that a simulated night shift decreases glucose tolerance and insulin sensitivity of healthy participants (14, 34, 70, 74-76). Thus, in a cross-over design, healthy lean and overweight participants followed a controlled 8-day laboratory protocol with a circadian alignment and –misalignment arm. Both postprandial glucose and –insulin excursions were higher after misalignment compared to alignment condition, both after one and after three days of misalignment, indicating slow adjustment of glucose tolerance upon circadian misalignment (34). Follow-up research found that the decreased glucose tolerance was not related to differences in beta-cell responsiveness but could mainly be attributed to a decrease in insulin sensitivity (75). Another circadian misalignment study suggested that it takes healthy participants at least two days to adapt to a simulated night shift. In this study, a meal test was performed under normal conditions (baseline), directly after a simulated night shift and two days after the night shift, and various metabolites were measured in response to the meal. Plasma free fatty acid levels were decreased in response to the night shift as compared to baseline whereas postprandial triglycerides were increased, and these values did not appear to return to baseline levels two days after the simulated night shift (77). Interestingly, decreased glucose tolerance upon experimentally induced circadian misalignment was also observed in individuals performing regular shift work, suggesting no long-term adaptive responses (74, 78), although it is unclear if the decrease in glucose tolerance is different in magnitude between shift workers and non-shift workers.

One aspect that complicates the interpretation of circadian misalignment studies is the loss of sleep that is associated with circadian misalignment, and sleep loss has also been reported to decrease insulin sensitivity and glucose tolerance in healthy volunteers (79-83). Interestingly, a parallel intervention study in two groups of healthy lean males undergoing either sleep deprivation (5 hours of sleep opportunity) or sleep deprivation plus circadian misalignment, showed significantly lower glucose tolerance

in the misalignment group (84). These findings indicate that the detrimental metabolic effects during circadian misalignment cannot be fully attributed to sleep loss (84).

Hence, eating and being active at a time that the body is not prepared for it impairs metabolic health beyond the effects of experienced sleep loss. Furthermore, the body does not adapt quickly to disturbed rhythms and does not become impervious to frequent challenges of changes in circadian rhythmicity, as is the case with shift-workers.

The potential of timed lifestyle interventions

Since several key metabolic processes have proven to be rhythmic, and disturbances in the timing of behavioural/environmental rhythms hamper metabolic health, it can be hypothesized that properly aligning the external behavioural and environmental rhythms with the circadian system is able to improve metabolic health. In this context, timing of lifestyle factors such as food (intake) and exercise have been given considerable attention over the past years.

Recent studies showed that both in the United States and in Europe energy consumption is distributed over the largest part of the day (5, 85). This means that these populations eat at time points that the body is not optimally prepared for it and also results in a lack of a true nocturnal fast. This in itself can be viewed as circadian misalignment (5, 85). Furthermore, since food intake is an important *Zeitgeber*, eating at the wrong time of day may desynchronize peripheral rhythms involved in metabolism from the central rhythm generated by the SCN, thereby leading to aberrant metabolic rhythmicity (3). Time restricted eating (TRE), i.e. consumption of the habitual diet within a limited time window (usually ≤12 hrs) without (deliberately) attempting to change the nutritional or caloric content of the diet, has been proposed to improve metabolic health by restoring fasting-feeding cycles allowing the body to utilize nutrient stores (86). Indeed, mice fed an *ad libitum* high fat diet, confined to a

limited period during the active phase (night time for mice), displayed an improved circadian rhythm in macronutrient utilization (87) as well as an improved glucose homeostasis as compared to non-time-restricted fed mice (87, 88). Additionally, time restricted feeding also protected mice against high-fat diet-induced weight gain, despite caloric intake being similar between both groups (87, 88).

In humans, restricting food intake of healthy overweight and obese adults to a self-selected time frame of 10-12 hrs per day for 16 weeks induced 3.8% weight loss, without any dietary recommendations (5). Later studies also reported that TRE was able to induce weight loss in overweight and obese adults (89-92). In overweight/obese males at risk for T2D, a 5-week fully supervised 6-hrs TRE (8AM-2PM) regime improved insulin sensitivity, β-cell responsiveness, blood pressure, oxidative stress and appetite as compared to the control group who ate in a 12-hrs eating time frame, despite similar caloric intake and meal frequency (93). Consistently, a 9-hrs TRE improved glucose tolerance and reduced fasting triglycerides in obese males at risk for T2D (94). In this study, it was also shown that the time period during which the 9 hours of eating was planned (early vs later in the day) had no effect on the beneficial outcome of TRE (94). Additionally, it has been found that a 6-hrs TRE for 4 days increased fat oxidation in overweight and obese adults as compared to energy consumption spread out over 12 hrs, even though total energy intake was similar between the two arms (95. A more recent randomized crossover study in overweight and obese men reported that a 5-day 8-hrs TRE regime reduced nocturnal glucose levels compared to spreading the intake of the same amount of calories over a 14-hrs timeframe (96). The main outcomes of recent human TRE studies, performed in overweight and obese participants, are summarized in table 1 and collectively show that restricting food intake to a limited time frame improves metabolic health in both rodent and humans.

Besides limiting the timeframe of caloric intake, TRE regimes often distribute food intake to the earlier part of the day, which in itself may confer metabolic benefits.

Thus, several studies report a greater weight loss efficacy of hypocaloric diets (97, 98), hypocaloric diets in combination with moderate exercise (99, 100) and bariatric surgery (101) in overweight and obese participants when the majority of calories are consumed earlier in the day, typically before 3 AM (97, 99, 101), even when total energy intake is similar between early and late eaters (98-102). Please note that these are not TRE studies, as not all energy intake was consumed before 3AM. Conversely, consuming more calories in the evening (103) or eating closer to the biological night (104) has been associated with higher adiposity compared to eating early during the day (103, 104). In an experimental setting, a study in young lean men delayed meal times with 5-hrs without changing meal content and found that circadian rhythms (measured using the constant routine protocol, see figure 2) in plasma glucose were delayed by the late meals without changes in rhythmicity of circulating insulin and triglycerides levels (105). In addition, healthy lean females eating lunch (47% of total energy intake) at 4.30 PM vs. 1 PM for a week displayed a decreased glucose tolerance, resting energy expenditure, and fasting carbohydrate oxidation (106). Several randomized controlled trials also suggest that skipping breakfast, thereby consuming the majority of calories later during the day, worsens metabolic health in healthy adults (107, 108), although not all studies agree (109, 110). A randomized crossover study in healthy lean and overweight adults compared the effect of omitting breakfast and eating a late night snack (at 10PM) with eating breakfast (at 8AM) and omitting the snack (111). The study found that the typical nocturnal switch to fat oxidation that was found in the breakfast arm was absent in the late snack arm, despite similar meals, fasting times and total energy expenditure (111). Moreover, when participants with T2D consumed a high caloric breakfast for 7 days an overall lower postprandial hyperglycaemia was found compared to consuming a high caloric dinner, even though total daily energy was equal (97). Hence, although there is still some controversy on the most optimal meal timing, accumulating evidence suggests that consumption of the majority of calories earlier in the day leads to metabolic benefits. This has also lead

to a recommendation issued by the American Heart Association (112), which was based on a meta-analysis of the effects of timing of food intake on metabolic health.

Table 1 Time-restricted eating studies performed in humans.

Study	Population	Eating hrs	TRE Time frame	Control arm	Period	EI	Weight loss	Outcome
Chow et al. 2020 (89)	Overweight and obese adults	8 h	Self-selected	Unlimited eating time	12 weeks	↓	Yes	Body Weight ↓ Lean mass ↓
Gabel et al. 2018 (90)	Obese adults	8 h	10AM-6PM	Habitual eating times	12 weeks	↓	Yes	Body weight ↓ Blood Pressure ↓
Gill and Panda. et al 2015 (6)	Overweight and obese adults	10-12 h	Self-selected	No	16 weeks	↓	Yes	Body Weight ↓ Sleep satisfaction ↑
Hutchinson et al. 2019 (94)	Men at risk for T2D	9 h	8AM-5PM vs 12PM-9PM	No	7 days	=	Yes, both arms	Glucose tolerance ↑ Fasting triglycerides ↓ Similar in both TRE interventions
Jamshed et al. 2019 (92)	Overweight and obese adults	6 h	8AM-2PM	Eating from 8AM-8PM	4 days	=	Yes	24-hrs glucose ↓
Parr et al. 2020 (96)	Overweight and obese males	7 h	10AM-5PM vs 7AM-9PM	Eating from 7AM-9PM	5 days	=	?	Nocturnal glucose ↓
Ravussin et al. 2019 (95)	Overweight and obese adults	6 h	8AM-2PM	Eating from 8AM-8PM	4 days	=	Yes	Energy expenditure = Fat oxidation ↑ Hunger ↓
Sutton et al. 2018 (93)	Men at risk for T2D	6 h	8AM-2PM	Eating from 8AM-8PM	5 weeks	=	No	Insulin sensitivity ↑ Blood pressure ↓ Oxidative stress ↓
Wilkinson et al. 2020 (91)	Adults at risk for T2D	10 h	Self-selected	No	12 weeks	↓	Yes	Body weight ↓ Blood pressure ↓ Atherogenic lipid ↓

↓ denotes a decrease, = denotes no change, ↑ denotes an increase, and ? denotes that there was no information provided on the variable.

Next to manipulating food intake, exercise is traditionally viewed as another powerful lifestyle intervention, since it increases metabolic flexibility and skeletal muscle- and whole body insulin sensitivity (113). Importantly, exercise acts as a *Zeitgeber* for skeletal muscle (114, 115) and it is tempting to suggest that timing of exercise could help to reset disturbances due to circadian misalignment. The importance of the skeletal muscle molecular clock in metabolism is illustrated by the finding that knockout of the Bmal gene in skeletal muscle of mice not only resulted in a disturbed skeletal muscle glucose homeostasis but also disrupted whole-body glucose homeostasis (116). In humans, both skeletal muscle cells and –tissue from lean and overweight donors have been shown to exhibit rhythms in genes involved in glucose homeostasis and insulin resistance (117). In mice, 4 weeks of involuntary exercise during the inactive phase (daytime) resulted in an altered rhythm of the skeletal muscle molecular clock (114). Moreover, in humans, an acute bout of exercise already modulated the rhythmicity of the expression of molecular circadian clock components in skeletal muscle tissue (115). To further examine the role of regular exercise training on the molecular clock, synchronized skeletal muscle cells obtained from people with a sedentary lifestyle and from athletes have been obtained, but these cells do not show any difference in rhythmicity of molecular clock components (118). However, it was shown that only skeletal muscle cells taken from athletes exhibited rhythmicity in NAMPT (nicotinamide phosphoribosyl transferase) and SIRT1 (sirtuin 1), factors involved in regulating cellular energy status, suggesting that regular exercise training may improve some rhythmicity of skeletal muscle energy metabolism (118). However, it should be noted that these studies have been done in synchronized primary myotubes and that culture conditions may affect the outcome.

Next to effects on skeletal muscle, exercise has also been suggested to have effects on whole-body circadian rhythmicity. Thus, an in vivo study in healthy young males showed that exercise affects the oscillations normally seen in melatonin levels, generated by the central clock (119, 120), whereas nightly bouts of cycle ergometry exercise resulted in a phase delay of the melatonin rhythm (119). Interestingly,

regularly breaking the sedentary time with light-intensity walks during daytime has been shown to improve the nocturnal glucose levels of overweight and obese T2D patients as compared to prolonged sitting (121). Based on these promising results, it can be anticipated that exercising at a particular time of day could be more or less effective with respect to the physiological and metabolic outcomes of exercise training. Indeed, mice exercising on a treadmill at the beginning of the active phase show a higher skeletal muscle carbohydrate- and ketone body utilization than mice exercising at the beginning of the rest phase, indicating that timing of exercise differentially affects metabolism (122). To date, only a few human studies have been performed that specifically looked into the effects of exercise timing on metabolic outcomes. In that context, timing of exercise can be interpreted as the clock time of day when exercise is performed, but also the time of exercise relative to the time of a meal. Thus, a study in obese insulin resistant adults showed that three short (6 x 1 minute) intense exercise bouts (walking incline) per day, immediately before breakfast, lunch and dinner, reduced postprandial and 24hrs glucose levels more effectively when compared to a single 30 minutes bout of moderate continuous exercise performed before dinner (123). Furthermore, a low intensity walk after a meal has been shown to decrease postprandial glucose excursions in healthy participants and participants with type 1 diabetes as compared to meals followed by inactivity (124). It should be noted that – despite similar meal times and content – differences in workload exist in the abovementioned studies. These differences in exercise intensity are likely to contribute to the different metabolic benefits observed, which hampers conclusions on the true effect of the timing of the exercise.

The effect of exercising at different clock times of day has been investigated in only a few human studies so far. It has been found that male cyclists have increased levels of insulin and cortisol in response to a morning cycling test as compared to cycling in the evening, and these changed hormone levels may also affect substrate metabolism

(125). In young lean participants, an acute 1-hour submaximal aerobic exercise test performed in the evening decreased glucose levels and led to a relatively higher glucose oxidation when compared to a similar exercise bout performed in the morning (126). Similar findings were reported in overweight men with T2D who performed high-intensity interval training (HIIT) either in the morning or in the afternoon, with more effective reductions in 24h blood glucose levels when exercise was done in the afternoon compared to morning HIIT (127). Taken together, rodent and human studies have convincingly showed rhythmicity in skeletal muscle metabolism and modifying these rhythms to improve metabolic health by means of (timed) exercise may be a promising future approach.

Conclusions

The human body possesses a circadian system that anticipates predictable disturbances in homeostasis. Both diurnal- and circadian rhythm studies show that also metabolic processes of healthy, lean adults fluctuate during the day. People with obesity-related metabolic disturbances, however, have shown an altered rhythm in glucose homeostasis as well as disturbances in (output of) the SCN, indicating a hampered circadian system. Moreover, experimentally-induced circadian misalignment by a rapid switch of the day and night deteriorates metabolic health of otherwise healthy volunteers. Combined, these findings indicate that metabolic disturbances are characterized and possibly caused by a disturbed circadian rhythm. The intimate relationship between the circadian system and metabolism also opens up new opportunities to improve metabolic health by means of reinforcement of the circadian rhythm. Both food intake and exercise have been shown to be important *Zeitgebers* for peripheral clocks in metabolic tissues, indicating that they can modulate the circadian rhythms in these tissues. Therefore, eating and exercising at a time that the body is prepared for it does not only lead to the appropriate homeostatic response, but also reinforces the circadian rhythm. Interventions aimed at improving

metabolic health by adjusting the timing of lifestyle factors have shown promising results, although the amount of human studies addressing this topic is still limited. Therefore, human studies investigating the effects of timed lifestyle interventions on metabolism, including the underlying mechanisms, are highly encouraged.

Competing Interests

The authors declare no competing interests

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Chapter 3

Amount and timing of physical activity in relation to sleep quality and duration in the general population: a cross-sectional analysis

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Chapter in preparation for publication

Chapter 4

A prolonged fast improves overnight substrate oxidation, without modulating hepatic glycogen in adults with and without NAFL: a randomized cross-over trial

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STUDY IMPORTANCE QUESTIONS

What is already known about this subject?

- Increasing overnight fasting time, as with time-restricted eating, has been shown to improve metabolic health and could be of therapeutic value in individuals with metabolic disturbances, including individuals with non-alcoholic fatty liver (NAFL).
- People with disturbed metabolism show a blunted increase in fat oxidation during the night.

What are the new findings in your manuscript?

- Acutely prolonging the overnight fast can improve overnight substrate oxidation, however these alterations are not mediated by changes in hepatic glycogen depletion.
- Despite fasting for 16 hours, the overnight carbohydrate oxidation of the NAFL group stayed relatively high compared to carbohydrate oxidation of the control group.

How might your results change the direction of research or the focus of clinical practice?

- Our results suggest that the beneficial metabolic effects of time restricted eating may be related to higher overnight fat oxidation, but indicate a minor role for hepatic glycogen fluctuations.

ABSTRACT

Objective: Increasing overnight fasting time seems a promising strategy to improve metabolic health in individuals with non-alcoholic fatty liver (NAFL). Mechanisms underlying the beneficial effects of fasting may be related to larger fluctuations in hepatic glycogen and higher fat oxidation. Here, we investigated whether prolonging an overnight fast depletes hepatic glycogen stores and improves substrate metabolism in individuals with NAFL and healthy lean individuals.

Methods: Eleven individuals with NAFL and ten control individuals participated in this randomized cross-over trial. After a 9.5-hour or 16-hour fast, hepatic glycogen was measured by ^{13}C-MRS and a meal-test was performed. Nocturnal substrate oxidation was measured with indirect calorimetry.

Results: Extending fasting time led to lower nocturnal carbohydrate oxidation and higher fat oxidation in both groups (intervention*time, $p<0.005$; for carbohydrate and fat oxidation). In both arms, the respiratory exchange ratio measured during the night remained higher in the NAFL group compared to the control group (population $p<0.001$). No changes were observed in hepatic glycogen depletion with a prolonged overnight fast in the NAFL or control group.

Conclusions: These results suggest that acutely prolonging the overnight fast can improve overnight substrate oxidation and that these alterations are not mediated by changes in hepatic glycogen depletion.

INTRODUCTION

In our current 24-hour society most people tend to spread their food intake over an interval of minimally 15 hours (1), resulting in the absence of a pronounced nocturnal fasted state whereby energy stores, i.e. fat and glycogen, are scarcely used. Thus, in the postprandial state, carbohydrates are stored in the liver as glycogen. In the transition from the fed to the fasted state, the body starts relying on hepatic glycogen stores to maintain normoglycemia, a necessity to fuel tissues that are dependent on glucose, including the brain. Hepatic glycogen serves as the main glucose source from around 4 to 28 hours after the last meal (2) and indeed hepatic glycogen declines with fasting (3-5). Such a reduction in hepatic glycogen is important for metabolic health since it triggers (molecular) metabolic adaptations that induce a fasting state and increase fat oxidation (6). Fat oxidation is important for insulin sensitivity since it decreases the level of triglycerides in oxidative tissues and thereby prevents impaired insulin signalling resulting from lipid accumulation (7-9). Indeed, studies in both healthy lean individuals and individuals who are overweight also show an increase in fat oxidation during an acute, prolonged fast (10-12). Conversely, the absence of nocturnal reductions in hepatic glycogen – due to food intake spread over 24-hours – could lead to the activation of metabolic processes that promote hepatic lipid storage (13), and could thereby also promote the development of non-alcoholic fatty liver (NAFL). Importantly, NAFL increases the risk for other metabolic diseases, such as type 2 diabetes and cardiovascular diseases (14-16). Recently, we showed that participants with prediabetes indeed do not show the typical fed-to-fasting cycle in substrate oxidation, when compared to young, healthy participants (17, 18). However, if this switch in substrate oxidation can be recovered by a prolonged overnight fast remains to be investigated.

In recent years, repetitively prolonging the overnight fast by limiting food intake to a pre-determined time window during the day, i.e. time-restricted eating (TRE), has received much attention as a possible intervention to improve metabolic health. In healthy participants who are overweight/obese, it has been shown that TRE improves insulin sensitivity and increases nocturnal fat oxidation (12, 19). However, the mechanisms

underlying these benefits are incompletely understood. Here, we hypothesize that the beneficial metabolic effects of TRE are at least partially related to a more pronounced, nocturnal fasting state reflected by higher hepatic glycogen depletion and a higher rate of fat oxidation. As a proof-of-concept, the aim of this study was to assess the change in hepatic glycogen overnight after an acute, 16-hour overnight fast compared to a 9.5-hour fast in participants with NAFL compared to age-matched healthy, lean participants.

METHODS

Eleven participants with NAFL and, as a control, ten age-matched healthy lean participants were measured in two separate randomized cross-over trials which were both conducted at Maastricht University Medical Center, the Netherlands, between January 2019 and May 2021. Both studies were approved by the Medical Ethical Committee of Maastricht University Medical Center. Research was performed in accordance with all relevant ethical regulations regarding human research participants. All participants signed an informed consent form prior to the start of the study. The studies are registered at clinicaltrials.gov (NCT03593343 and NCT04510155). Originally the three main outcomes in the study with NAFL participants were plasma β-hydroxybutyrate, *de novo* lipogenesis and hepatic glycogen. However, as hepatic glycogen was not significantly reduced by a prolonged overnight fast in NAFL participants, we wanted to investigate whether this was characteristic for individuals with NAFL and therefore, a second study was performed in age-matched healthy lean participants with the aim to compare hepatic glycogen and substrate oxidation between the two groups (primary and secondary aim respectively). Here, we report hepatic glycogen and substrate oxidation results of both studies.

Participants

For both studies, males and postmenopausal females between 45 – 75 years were eligible for participation. In the NAFL group, participants were eligible when BMI was 27 – 38 kg/m^2 and liver fat content ≥5%, and in the control group BMI was 18 – 25 kg/m^2 and liver

fat content <5%. Liver fat content was determined prior to inclusion using ^{1}H-MRS, as described elsewhere (20). Exclusion criteria were: engagement in exercise for more than 2 hours per week, unstable body weight (weight loss or gain more than 3 kg in 3 months preceding enrollment), consumption of more than 2 alcoholic units per day, smoking more than 5 cigarettes per day, contra-indication for MRI, use of medication known to interfere with the outcome parameters, and diagnosed with diabetes or other active diseases. Randomization was performed with the use of computer-generated random numbers. Following inclusion, a fasted blood sample was analyzed and body composition was determined using a BodPod (Cosmed©, Rome, Italy) to characterize the participant groups. Participant characteristics are shown in **table 1.** Flowcharts of participant enrolment are depicted in SM Fig. 1. and SM Fig. 2.

Table 1: Participant characteristics

	Control (n=10)	**NAFL (n=11)**	**P value**
Age (years)	64 ± 7	62 ± 9	0.705
Sex (f/m)	3/7	3/8	0.918
Weight (kg)	70.4 ± 11.4	94.0 ± 10.5	< 0.001
BMI (kg/m^2)	23.7 ± 2.0	30.6 ± 2.7	<0.001
Body fat (%)	29.0 ± 9.6	38.7 ± 7.3	0.018
Plasma glucose (mmol/L)	5.3 ± 0.4	5.8 ± 0.6	0.045
ALT (U/L)	24 ± 4	36 ± 14	0.029
AST (U/L)	24 ± 5	29 ± 8	0.128
Intrahepatic fat content (% weight/ weight)	1.3 ± 1.1	12.9 ± 7.8	< 0.001

Data presented as mean ± SD. A p-value < 0.05 was considered statistically significant.

Research Design

To study the acute metabolic effects of a 16-hour fast versus a 9.5-hour fast, participants stayed at the research facility for two consecutive days on two separate occasions. Two days prior to each stay, participants were instructed to refrain from physical exercise and alcohol consumption. The two intervention arms (16-hour fasting protocol and 9.5-hour fasting protocol) were separated by a 2 – 6-week wash-out period during which participants were instructed to maintain their habitual lifestyle. Total food intake in the two protocols was identical, only the timing was different.

On day 1, participants stayed in the metabolic research unit where they received standardized meals. Energy intake was 10.7 MJ in the NAFL group and 8.2 MJ in the control group and was based on the estimated average energy requirement for both populations, which was obtained by calculating the energy needs for the first five participants in the NAFL group and in the control group with the Harris and Benedict formula. Breakfast consisted of ~20 percent of total energy intake (En%), lunch ~30 En%, snack ~10 En%, and dinner ~40 En%. Daily macronutrient composition was ~57 E% carbohydrates, ~28 E% fat and, ~14 E% protein. Breakfast was given at 8 am and lunch at 12 pm in both protocols. In the 16-hour fasting protocol, the evening meal and a snack were given simultaneously at 4.30 pm. In the 9.5-hour fasting protocol, a snack was provided at 4.30 pm and the evening meal was provided at 11 pm (**figure 1**). On day 1, hepatic glycogen was measured using ^{13}C-MRS at 2 pm. In the evening of day 1, at 9 pm, participants entered the respiration chamber. For both interventions, participants were instructed to go to sleep at 11.30 pm. The following morning, on day 2, participants were woken up at 6 am and hepatic glycogen was measured at 6.30 am. Afterwards, a meal test was started at 8 am, after which the study protocol ended.

Due to the nature of the study, neither volunteers nor investigators were blinded to the study interventions. The quality of the data was checked without knowledge on intervention allocation.

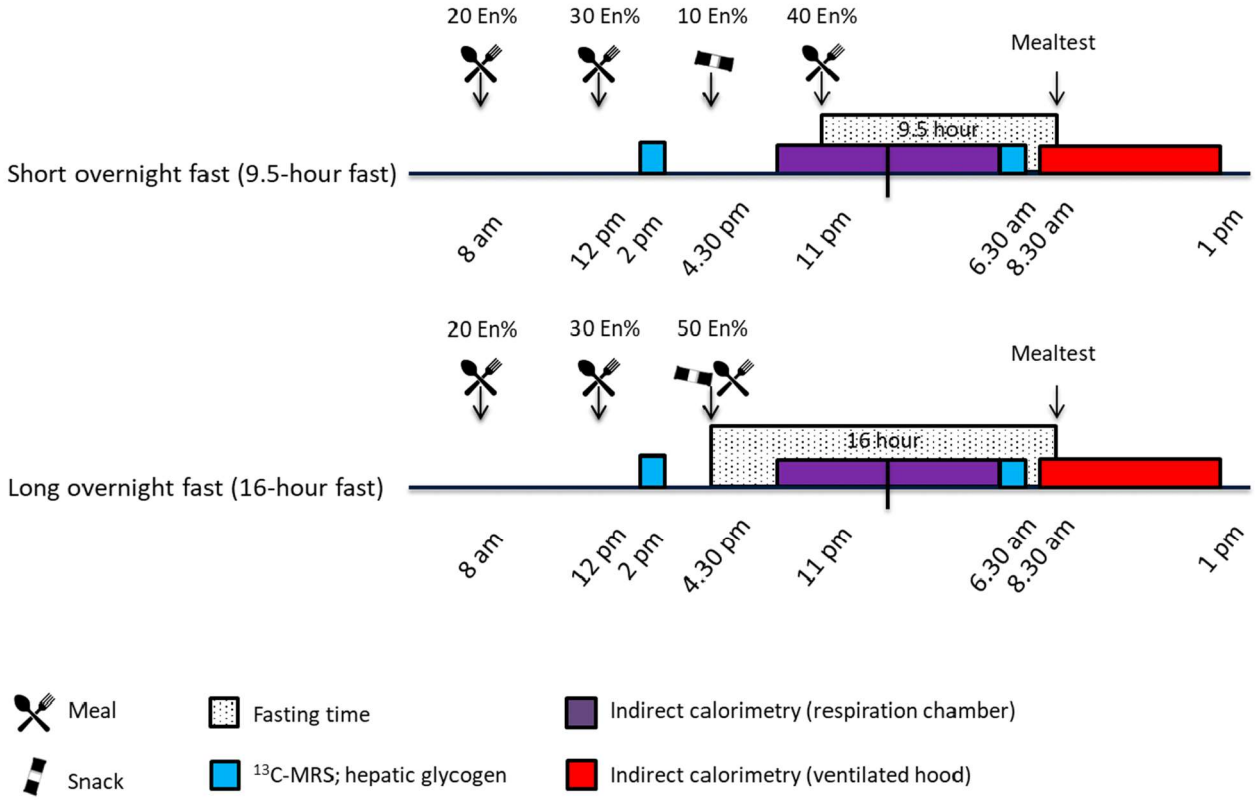

Figure 1: Study protocol. Participants participated in two overnight fasting protocols (9.5-hour fast and 16-hour fast). En%, percent of total energy intake.

Measurement of hepatic glycogen and liver volume

Hepatic glycogen was measured using ^{13}C-MRS. All ^{13}C-MRS experiments were performed on a 3T MR system (Achieva 3T-X Philips Healthcare, Best, Netherlands) by using a 21x24cm ^{13}C-quadrature detection surface coil (RAPID Biomedical GmbH, Germany). Spectra were acquired with the participant lying in the prone position (FID; TR: 280ms; NSA: 4096) using a block pulse, calibrated to achieve a 90-degree excitation angle at a distance of 8 cm from the coil. Data analysis was performed with an in-house developed MATLAB (MATLAB 2018b, The MathWorks, Inc., Natick, Massachusetts, United States) script. Phase-correction, baseline correction and frequency alignment were applied in the morning and afternoon spectra from the same individual simultaneously with the aim to minimize differences between spectra and therefore ensure identical pre-processing of both spectra. The area under the curve (AUC) of the glycogen signal was determined by

integration of ± 5 PPM around the C1 doublet of glycogen at 100.5 PPM. A coil sensitivity map was determined in a phantom with a big cylindrical phantom, filling the curved coil completely and containing a saturated solution of glucose and NaCl at a physiological concentration. The sensitivity map was generated with two-dimensional chemical shift imaging (multiple slices) and this sensitivity profile was taken into account by manually segmenting the liver on cross-sectional MRI images in a custom written MATLAB script and multiplying the number of pixels in the liver with the respective sensitivity (weighting of voxels based on the sensitivity map). Thereafter, the AUC of the glycogen signal was divided by the weighted number of liver pixels. Coil loading was assumed to be identical between the measurements within the same individuals. Therefore, even without correction for coil loading, the signal difference within an individual is directly proportional to the relative difference in glycogen concentration. Subsequently, relative changes in glycogen were calculated and given as percentage changes ((hepatic glycogen $_{6.30\ am}$ - hepatic glycogen $_{2\ pm}$)/ hepatic glycogen $_{2\ pm}$ * 100). Liver volume was measured directly after the hepatic glycogen measurements by MRI and total glycogen was calculated as glycogen content multiplied by liver volume. Analyses were performed manually on cross-sectional MRI images in MATLAB. Due to practical problems, ^{13}C-MR spectra of hepatic glycogen and liver volume MRI images could not be assessed at all time points for every participant. As such, changes in hepatic glycogen were calculated for 10 participants in the NAFL group, and liver volume for 8 participants in the NAFL group.

Respiration chamber measurement

The respiration chamber was used to measure nocturnal energy expenditure and substrate utilization. The respiration chamber is a small room with a bed, toilet, television, computer, and access to water, in which oxygen consumption and carbon dioxide production are measured continuously in sampled room air by indirect calorimetry (Omnical, Maastricht Instruments, Maastricht, The Netherlands (21)). From the respiration chamber data, sleeping metabolic rate (SMR) was determined by calculating the lowest 3 hours of energy expenditure during the night using the Weir equation (22).

Furthermore, substrate oxidation and energy expenditure were calculated using the Brouwer equation (23). Protein oxidation was estimated to be 12.4% of total energy expenditure using the Weir equation (22). The respiratory exchange ratio (RER) was calculated by dividing carbon dioxide production by oxygen consumption.

Meal test

After the MRS measurements in the morning of day 2, a meal test was performed. To this end, a high-energy breakfast was provided to the participants at 8.30 am. This meal entailed bread-based food items containing a total energy content of ~3.4 MJ for the NAFL group and ~3.1 MJ for the control group, about 38% of the estimated average energy requirement for both populations. The macronutrient composition of the breakfast was similar for both groups and was ~55 E% carbohydrates, ~29 E% fat, and ~15 E% protein. Blood draws and indirect calorimetry measurements were performed 30 minutes before the meal and at 30, 60, 120, 180, and 240 minutes after the meal was given. Indirect calorimetry measurements were performed with a ventilated hood system (Omnical, Maastricht Instruments, Maastricht University). Substrate oxidation and energy expenditure were determined using the Brouwer equation (23) and protein oxidation was estimated as 12.4% of total energy expenditure based on the pre-meal measurement.

Biochemical analysis

EDTA-plasma glucose (Horiba, Montpellier, France) and free fatty acids (WAKO, Neuss, Germany) levels were measured colorimetrically using a Cobas Pentra C400 analyzer (Horiba, Montpellier, France). Triglyceride levels were measured colorimetrically in EDTA-plasma (Roche, Mannheim, Gemanry) using Roche/Hitachi Cobas c systems. Insulin levels were measured in EDTA-plasma using an ELISA (Crystal Chem, Els Grove Village, USA). All samples from one participant were analysed within the same run.

Sample size

Sample size was determined based on demonstrating differences in overnight change in hepatic glycogen upon 9.5-hour and 16-hour fasting. Based on previous research (3), a difference of 75 mmol/L with a SD of the difference of 75 mmol/L was expected between 9.5-hour and 16-hour fasting. Using an alfa of 0.05 and power of 0.8, 10 participants were required in each study group.

Statistical analyses

Participant characteristics are expressed as mean ± SD, intervention effects are expressed as mean ± SEM. Differences in baseline characteristics between control and NAFL participants were compared with independent sample t-tests. Differences between study populations in hepatic glycogen utilization, metabolic meal test response and nocturnal substrate oxidation were tested using the linear mixed model (LMM) procedure. The LMMs included random effects to account for differences between participants, and the following fixed effects: intervention (9.5-hour versus 16-hour fast) and population (NAFL versus control). In addition, all LMMs included variables to test for interaction effects. Furthermore, the AUC was calculated to assess the meal test response. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 27.0 software (IBM Corp, New York, USA).

RESULTS

Respiration chamber

To test if a prolonged overnight fast affected nocturnal energy and substrate metabolism, participants were studied in our respiration chambers. As expected, SMR was higher in the NAFL group compared to the control group (population, $p = 0.003$), which can be attributed to the higher body weight and higher fat-free mass. Furthermore, SMR was higher with the 9.5-hour fast versus 16-hour fast (intervention, $p < 0.001$), probably due to the residual diet-induced thermogenic effect of the last meal. Interestingly, SMR showed a tendency for a population*intervention interaction effect ($p = 0.053$), which may indicate a higher SMR upon 9.5 hours compared to 16 hours of fasting in the NAFL group versus the control group (**figure 2A**).

RER measured during the night was higher in the NAFL group compared to the control group, (population, $p < 0.001$, **figure 2B**). Furthermore, in both groups, there was a higher RER with the 9.5-hour compared to the 16-hour fast (intervention, $p < 0.001$, **figure 2B**). To assess if RER was different during the course of the night, we divided the night into 3 time slots (i.e., 0.30 – 2 am, 2 – 3.30 am, 3.30 – 5 am) and added an additional variable of "time" into the linear mixed model. We found that both groups similarly exhibited a change in RER over the night in response to the different fasting protocols (intervention*time, $p = 0.002$, **figure 2C**). In line with the RER, carbohydrate oxidation and fat oxidation exhibited different responses to the fasting protocols (carbohydrate oxidation: intervention*time, $p = 0.003$, **figure 2D**; fat oxidation: intervention*time, $p = 0.002$, **figure 2E**). The difference in substrate oxidation over time between the two fasting protocols was characterized by higher carbohydrate oxidation and lower fat oxidation with the 9.5-hour fast compared to the 16-hour fast, which was most pronounced from 2 - 3.30 am and 3.30 - 5 am. For the relative contribution of carbohydrate and fat oxidation to total energy expenditure see SM Fig. 3.

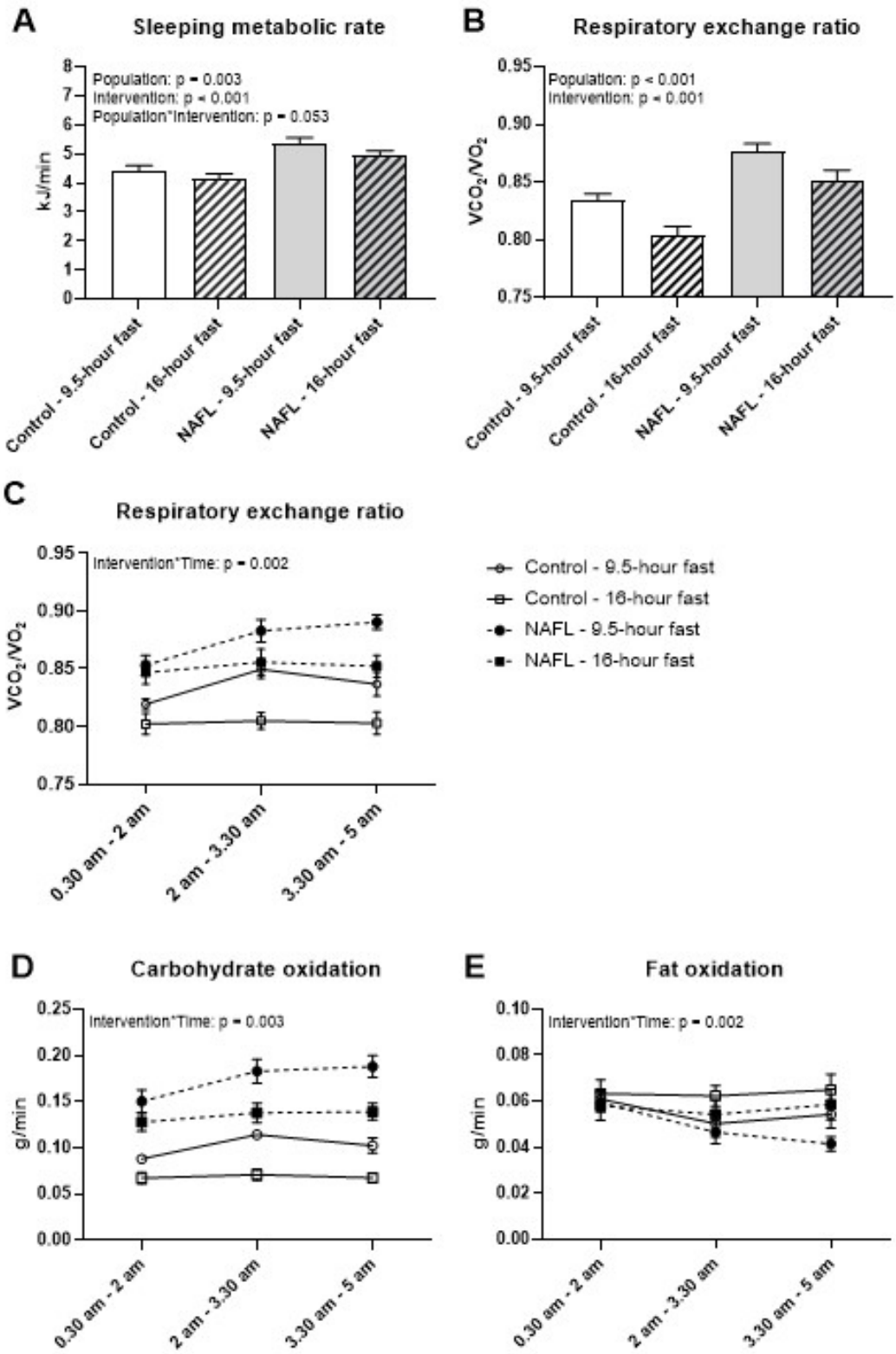

Figure 2: Sleeping metabolic rate and nocturnal substrate oxidation during 9.5 hours and 16 hours overnight fasting. A: Sleeping metabolic rate; **B**: Overnight respiratory exchange ratio during a 9.5-hour and 16-hour fast; **C**: Respiratory exchange ratio over the course of the night in the 9.5-hour and 16-hour fasting condition; **D**: Carbohydrate oxidation over the course of the night in the 9.5-hour and 16-hour fasting condition; **E**: Fat oxidation over the course of the night in the 9.5-hour and 16-hour fasting condition. For the control group, n = 10; for the NAFL group, n = 11. Data are presented as mean ± SEM.

Hepatic glycogen and liver volume measurement

Next, we examined if prolonged fasting affected hepatic glycogen levels. No differences in hepatic glycogen content were observed in both the NAFL and the control group in response to 16 hours fasting as compared to 9.5 hours fasting (**figure 3A**). Furthermore, no differences were observed in liver volume, or total hepatic glycogen in both the NAFL and the control group in response to 16 hours of fasting as compared to 9.5 hours of fasting (**figure 3B and 3C**).

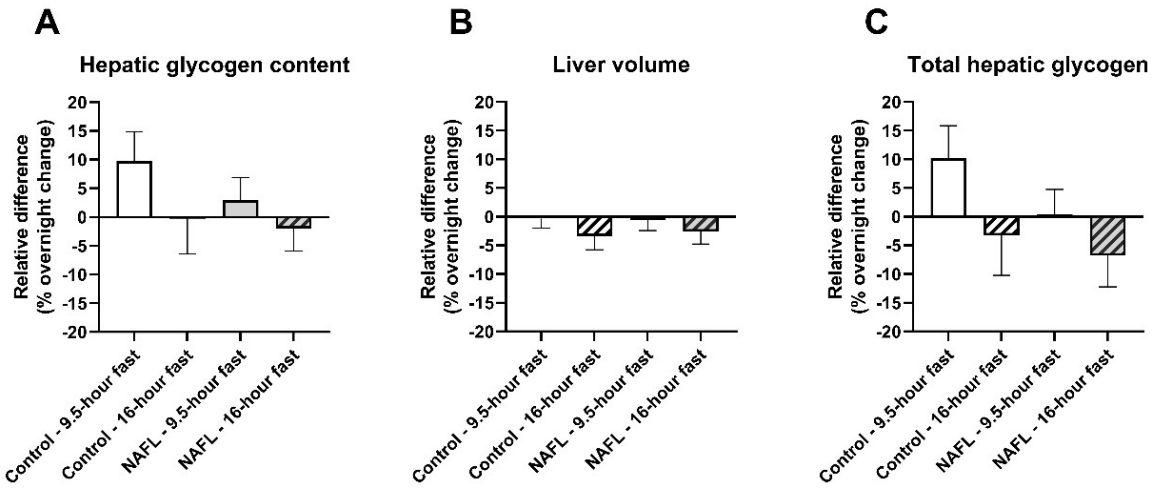

Figure 3: Hepatic glycogen upon 9.5 hours and 16 hours overnight fasting. A: Relative change in hepatic glycogen, not corrected for liver volume, from 2 pm to 6.30 am the next morning; **B**: Relative change in liver volume from 2 pm to 6.30 am the next morning; **C**: Relative change in total hepatic glycogen, corrected for liver volume, from 2 pm to 6.30 am the next morning. For A: the control group, n = 10; the NAFL group, n = 10; for B and C: the control group, n = 10; for the NAFL group, n = 8. Data are presented as mean ± SEM.

Meal test

To test if an acute prolonged overnight fast affects the postprandial response to a breakfast meal, we performed a standardized meal test. Interestingly, fasting glucose levels appeared to be higher with 16 hours fasting compared to 9.5 hours fasting, and this

difference was more pronounced in the control group compared to the NAFL group (population*intervention, p = 0.043, **table 2**). Furthermore, fasting free fatty acid levels were higher in the NAFL group compared to the control group, especially after 16 hours of fasting, although this did not reach statistical significance (population*intervention, p = 0.096, **table 2**). No intervention or interaction effects were found for insulin and triglycerides, although insulin and triglyceride levels were higher in the NAFL group compared to the control group (population, p < 0.001 and p = 0.010, respectively). There was a tendency for an interaction effect for fasting RER (population*intervention, p = 0.063, **table 2**). Whereas RER was lower after 16 hours of fasting compared to 9.5 hours of fasting in the NAFL group, the opposite change tended to occur in the control group. No population or intervention effects were observed for RER.

Furthermore, when calculating the AUC of the postprandial response, the AUC of insulin was significantly higher in the NAFL group compared to the control group (population, p = 0.001, **figure 4B**). In both groups, the AUC of triglycerides were similarly increased with the 16-hour fast compared to the 9.5-hour fast (intervention, p = 0.014, **figure 4D**). In addition, the AUCs of triglycerides were higher in the NAFL group compared to the control group (population, p = 0.005, **figure 4D**). The AUC of carbohydrate oxidation during the meal test was higher in the NAFL group than in the control group (population, p = 0.018, **figure 4F**). There were no other significant differences in metabolites or substrate oxidation between the two populations or in response to the fasting protocols.

Table 2. Postprandial metabolites and substrate oxidation

		Control		NAFL		P-value		
		9.5-hours	**16-hours**	**9.5-hours**	**16-hours**	**Pop**	**Int**	**Pop*Int**
Glucose (mmol/l)	**Fasting**	5.5 ± 0.2	5.8 ± 0.2	5.8 ± 0.1	5.9 ± 0.2	0.409	0.017	0.043
	AUC	1555 ± 64	1586 ± 91	1650 ± 70	1649 ± 66	0.428	0.681	0.649
Insulin (mU/l)	**Fasting**	4.0 ± 0.9	4.0 ± 0.8	18.8 ± 2.7	16.6 ± 2.9	<0.001	0.224	0.224
	AUC	14029 ± 1814	12891 ± 2161	24304 ± 2209	24237 ± 2064	0.001	0.364	0.419
FFA (umol/l)	**Fasting**	498 ± 67	491 ± 45	522 ± 39	657 ± 48	0.118	0.130	0.096
	AUC	46057 ± 8091	41894 ± 4750	48624 ± 2764	54635 ± 4103	0.162	0.857	0.328
Triglycerides (mmol/l)	**Fasting**	1.4 ± 0.2	1.6 ± 0.2	2.4 ± 0.3	2.5 ± 0.2	0.010	0.144	0.487
	AUC	414 ± 51	452 ± 65	680 ± 67	754 ± 71	0.005	0.014	0.396
RER (VCO_2/VO_2)	**Fasting**	0.783 ± 0.010	0.798 ± 0.007	0.814 ± 0.010	0.800 ± 0.015	0.25	0.919	0.063
	AUC	206 ± 2	205 ± 1	207 ± 2	206 ± 2	0.499	0.460	0.962
Carbohydrate oxidation (g/min)	**Fasting**	0.05 ± 0.01	0.06 ± 0.01	0.10 ± 0.01	0.08 ± 0.02	0.031	0.989	0.051
	AUC	34 ± 2	33 ± 2	43 ± 3	42 ± 3	0.018	0.410	0.780
Fat oxidation (g/min)	**Fasting**	0.07 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.07 ± 0.01	0.332	0.991	0.132
	AUC	12 ± 1	13 ± 1	14 ± 1	14 ± 1	0.192	0.525	0.919
Energy expenditure (kJ/min)	**Fasting**	4.2 ± 0.2	4.3 ± 0.2	5.3 ± 0.2	5.2 ± 0.2	0.001	0.939	0.102
	AUC	1227 ± 51	1229 ± 47	1488 ± 44	1476 ± 47	0.001	0.553	0.443

Data represented as mean ± SEM. Results considered statistically significant if $p < 0.05$. Pop: population; Int: intervention; RER: respiratory exchange ratio

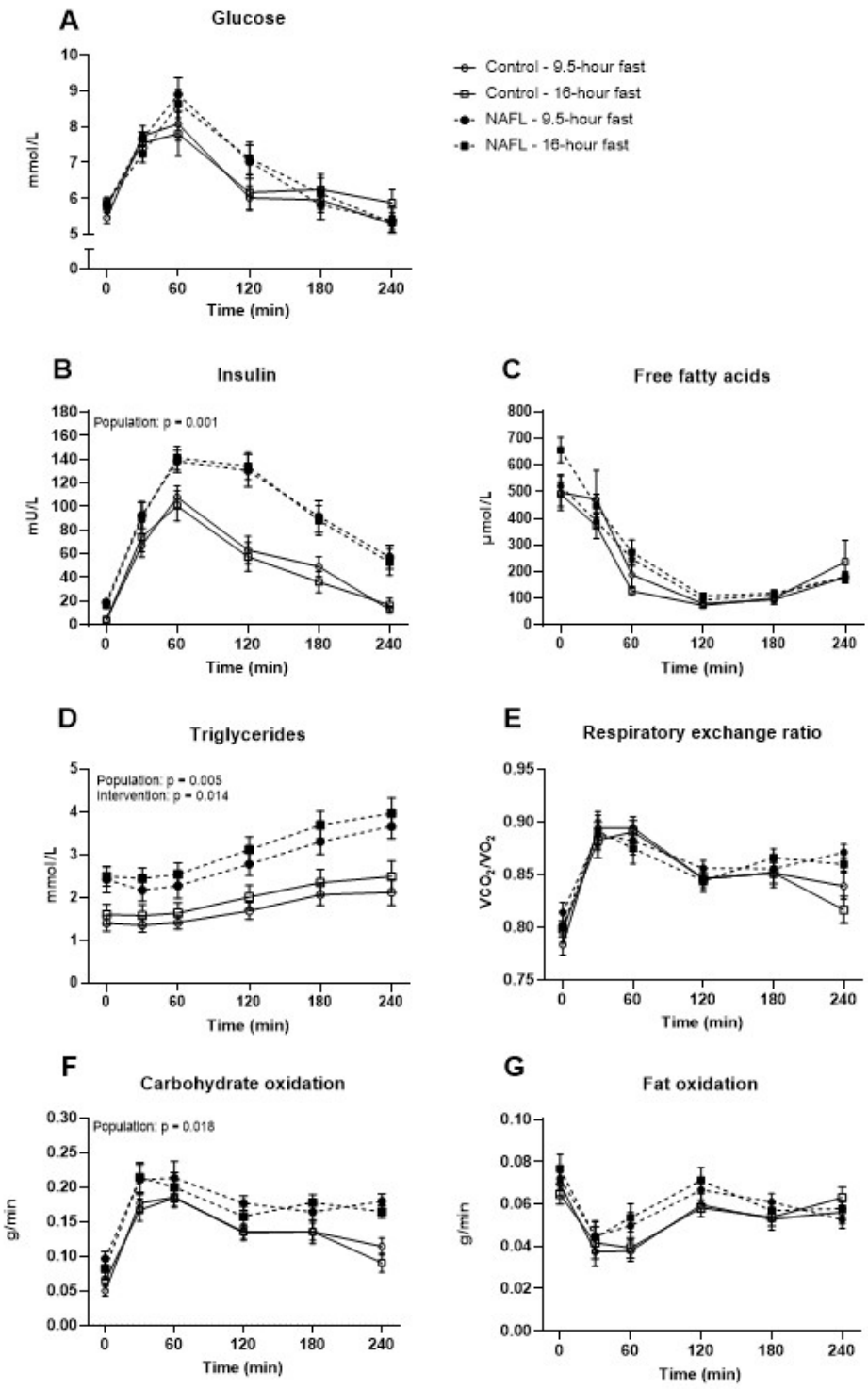

Figure 4: Metabolites and substrate oxidation during the meal test after 9.5 hours and 16 hours overnight fasting. A: Glucose; **B**: Insulin; **C**: Free fatty acids; **D**: Triglycerides; **E**: Respiratory exchange ratio; **F**: Carbohydrate oxidation; **G**: Fat oxidation upon a breakfast meal after 9.5 hours and 16 hours of fasting. For the control group, n = 10; for the NAFL group, n = 11. Data are presented as mean ± SEM.

DISCUSSION

Time-restricted eating has previously been shown to improve metabolic health in individuals with prediabetes, even in the absence of weight loss (24). Since hepatic glycogen is important in providing energy during (prolonged) fasting, we hypothesized that the beneficial effects may at least partially be due to stronger fluctuations in hepatic glycogen. Here we showed that prolonging the overnight fast by 6.5 hours for one night did not affect hepatic glycogen depletion in individuals with NAFL and healthy control participants when 24-hour energy intake was identical in the two conditions. Prolonging the overnight fast did have acute effects on nocturnal whole-body substrate oxidation in both groups, but did not affect the metabolic response to a breakfast meal.

In healthy young individuals, substrate oxidation switches to higher fat oxidation and lower carbohydrate oxidation in response to fasting, such as during the night (17). We recently showed that this switch to nocturnal fat oxidation is blunted in individuals with prediabetes (18). In the current study, extending fasting time resulted in lower carbohydrate oxidation and higher fat oxidation during the night in both the NAFL group and an age-matched, healthy control group, which was expected. However, although the effect of extending fasting time on substrate oxidation seemed similar in both groups, the absolute levels of fat oxidation remained lower in the NAFL group compared to the control group. Thus, even with a longer fasting time, there appeared to be a preference for carbohydrate oxidation and a lack of the typical fasting state with high rates of fat oxidation in the NAFL group. As such, in the NAFL group, the 16-hour fast decreased the RER to a level similar to the RER seen in the control group during the 9.5-hour fasting protocol. Therefore, these data indicate that the high RER in the NAFL group can be decreased by prolonging the fasting with a few hours. Examining substrate oxidation during three phases of the night also reveals that a late evening meal has effects on substrate metabolism during the whole night and in fact that during the night carbohydrate oxidation is gradually increasing, illustrating postprandial effects that last until the early hours of the next morning.

Since higher fat oxidation with fasting can be triggered by hepatic glycogen depletion, we hypothesized that a longer overnight fast would result in lower hepatic glycogen levels and that this effect would be more pronounced in the control group compared to the NAFL group. However, a 16-hour overnight fast did not result in an altered hepatic glycogen content compared to a 9.5-hour fast. Interestingly, the 16-hour fast appeared to decrease hepatic glycogen content as expected, whereas hepatic glycogen was increased with the 9.5-hour fast, irrespective of the study population, although these tendencies did not reach statistical significance. To investigate the fasting-induced change in hepatic glycogen, scans were performed at 2 pm and 6.30 am. In contrast to previous studies that investigated the effect of fasting on hepatic glycogen content (3, 4, 25), we here aimed to investigate if restricting food intake to a shorter time period – without restricting total energy intake – affects hepatic glycogen. Therefore, we kept the total carbohydrate and energy intake consumed between the afternoon and the morning glycogen scans identical in both conditions, and changed solely the timing of the meals. It has been shown previously that (prolonged) overnight fasting leads to reduced hepatic glycogen content in healthy individuals who are overweight and patients with type 2 diabetes (3-5). Reductions ranging from -19% to -55% in healthy lean individuals and -18% to -40% in patients with type 2 diabetes have been reported compared to postprandial levels, depending on the time of fasting (10 hours to 17 hours). In contrast to these findings, we did not find a reduction in hepatic glycogen levels in the morning when extending the fasting time, neither in individuals with NAFL nor in the control group. Interestingly, glycogen levels even slightly increased with 9.5 hours of fasting, an effect that was reversed with the 16-hour fast. This finding was most apparent in the control group but also occurred in the NAFL group albeit to a lesser extent. The timing of the hepatic glycogen measurements probably underlies these findings. The glycogen scans were performed at fixed timepoints in both study arms to avoid circadian influences on glycogen and – as explained above – to allow for the same caloric intake during the day. To this end, hepatic glycogen levels could not be determined at peak levels (4 to 5 hours

after a meal high in calories and carbohydrates), as this time point would be different for the two study arms. Rather, glycogen levels were determined at 2 pm, after a light lunch, in both arms. Therefore, 50% of daily energy intake (carbohydrate-rich snack and evening meal) was still to be ingested after the glycogen scan and this may have contributed to the fact that glycogen levels did not decline 'overnight' (i.e the difference between 2 pm and 6.30 am). This did however not affect the comparison of change in glycogen upon 9.5h vs. 16h fasting, as in both arms baseline hepatic glycogen levels were determined at 2pm.

Combining the results on substrate oxidation and hepatic glycogen, we found that overnight carbohydrate oxidation remained high in the NAFL group, even with prolonged fasting, whereas hepatic glycogen stores appeared unchanged. This raises the question what the source is for the glucose that is being oxidized. Next to glycogen, increased hepatic gluconeogenesis might be used as a means to provide glucose for carbohydrate oxidation in the NAFL group. Studies reporting increased rates of gluconeogenesis, but similar glycogenolysis in NAFL compared to individuals without NAFL support this hypothesis (3, 26, 27). An increase in gluconeogenesis could be driven by increased substrate availability (e.g. glycerol, lactate and pyruvate) and upregulation of gluconeogenic enzymes. Increased availability of pyruvate has been shown in NAFL, together with increased pyruvate carboxylase flux, driving substrate to the gluconeogenic pathway (26). Furthermore, NAFL is associated with increased adipose tissue insulin resistance (28), which can contribute to increased glycerol flux and thereby provide gluconeogenic substrates. Interestingly, extending overnight fasting time did reduce nocturnal carbohydrate oxidation in NAFL and controls to the same absolute extent – in parallel with non-significant, but similar changes in glycogen depletion – but carbohydrate oxidation remained elevated in NAFL. This may suggest that especially gluconeogenesis is not responding to the acute extension of fasting time and higher overnight gluconeogenesis in the NAFL group. However, since we did not assess gluconeogenesis in our study this requires further study.

In response to the meal test, we found postprandial insulin and triglycerides to be higher in the NAFL group compared to the control group, which has been reported previously (29). However, the effects of prolonging the overnight fast on fasting- and postprandial metabolites and substrate oxidation were limited. This lack-of-effect could indicate that an acute change in fasting time does not affect postprandial substrate metabolism. However, beneficial metabolic effects do occur when the overnight fast is repetitively prolonged, as with time-restricted eating (TRE). Indeed, 7 days of limiting food intake to a 9-hour time window has been reported to decrease the postprandial glucose response to a mixed-nutrient liquid test meal in males at risk for type 2 diabetes (30). However, a study that examined the effect of a 5-day 8-hour TRE regime in males with overweight or obesity, only found the glycaemic response to be decreased in response to a lunch meal and not in response to a breakfast meal (31). Combined, these studies indicate that prolonging the overnight fast repetitively can beneficially affect the glycaemic response to a test meal, but that the timing- and nutritional composition of the test meal may affect this response.

CONCLUSION

In conclusion, we found that acutely prolonging the overnight fast reduced carbohydrate oxidation and increased fat oxidation during the night in both individuals with NAFL and healthy control participants. Interestingly, despite fasting for 16 hours, the overnight carbohydrate oxidation of the NAFL group stayed relatively high compared to carbohydrate oxidation of the control group. However, we did not find changes in overnight hepatic glycogen depletion. These results suggest that a prolonged fast can improve overnight substrate oxidation, but this improvement is not mediated by changes in hepatic glycogen depletion.

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AUTHORS CONTRIBUTIONS

KR, AV, CA, HP, LL, PS, VS designed research; KR, AV, CA conducted research; KR, AV, CA, JM, EK, PV analyzed data or performed statistical analysis; KR, AV, CA, HP, LL, PS, VS wrote paper; VS had primary responsibility for final content. All authors have read and approved the final manuscript.

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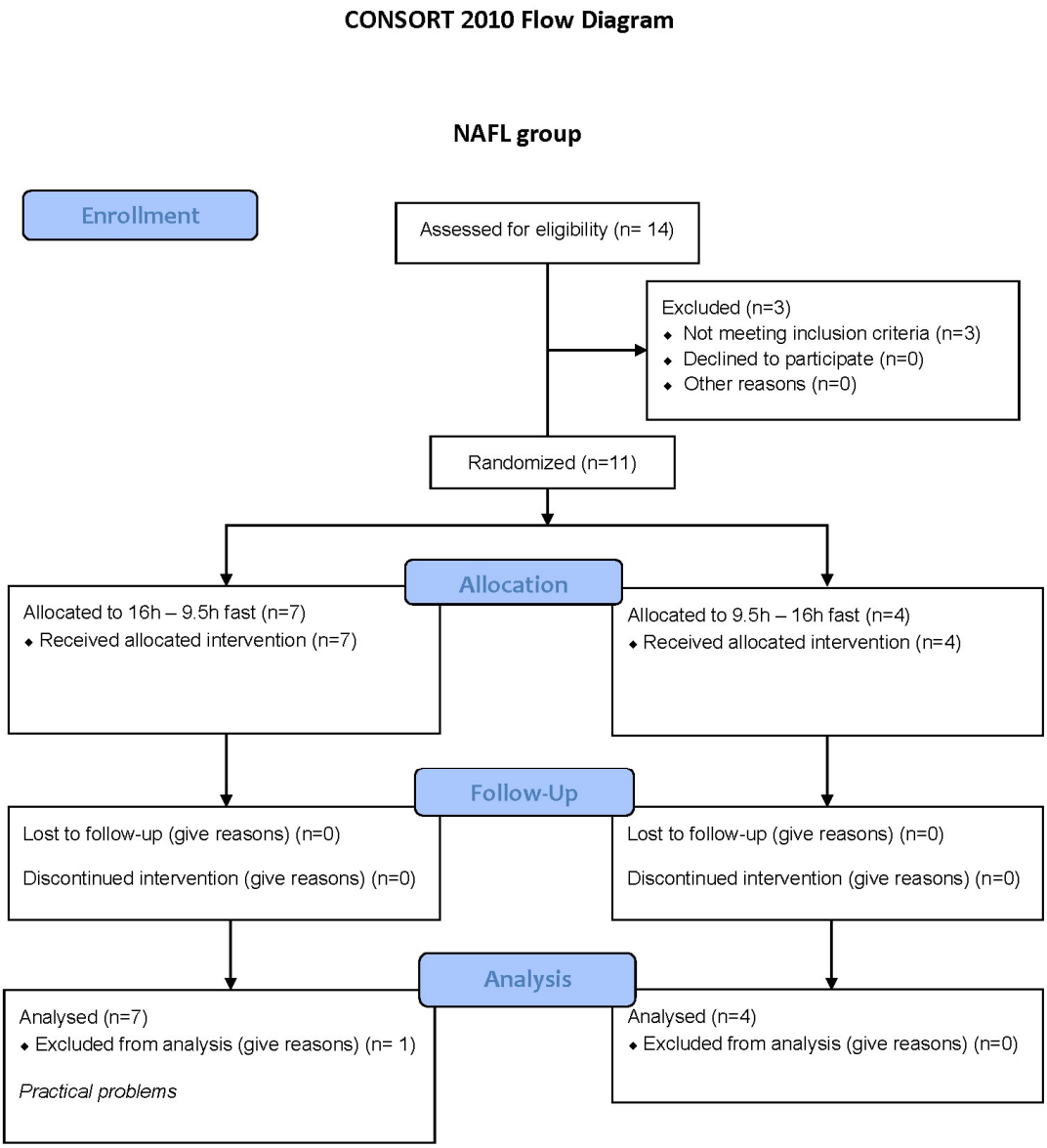
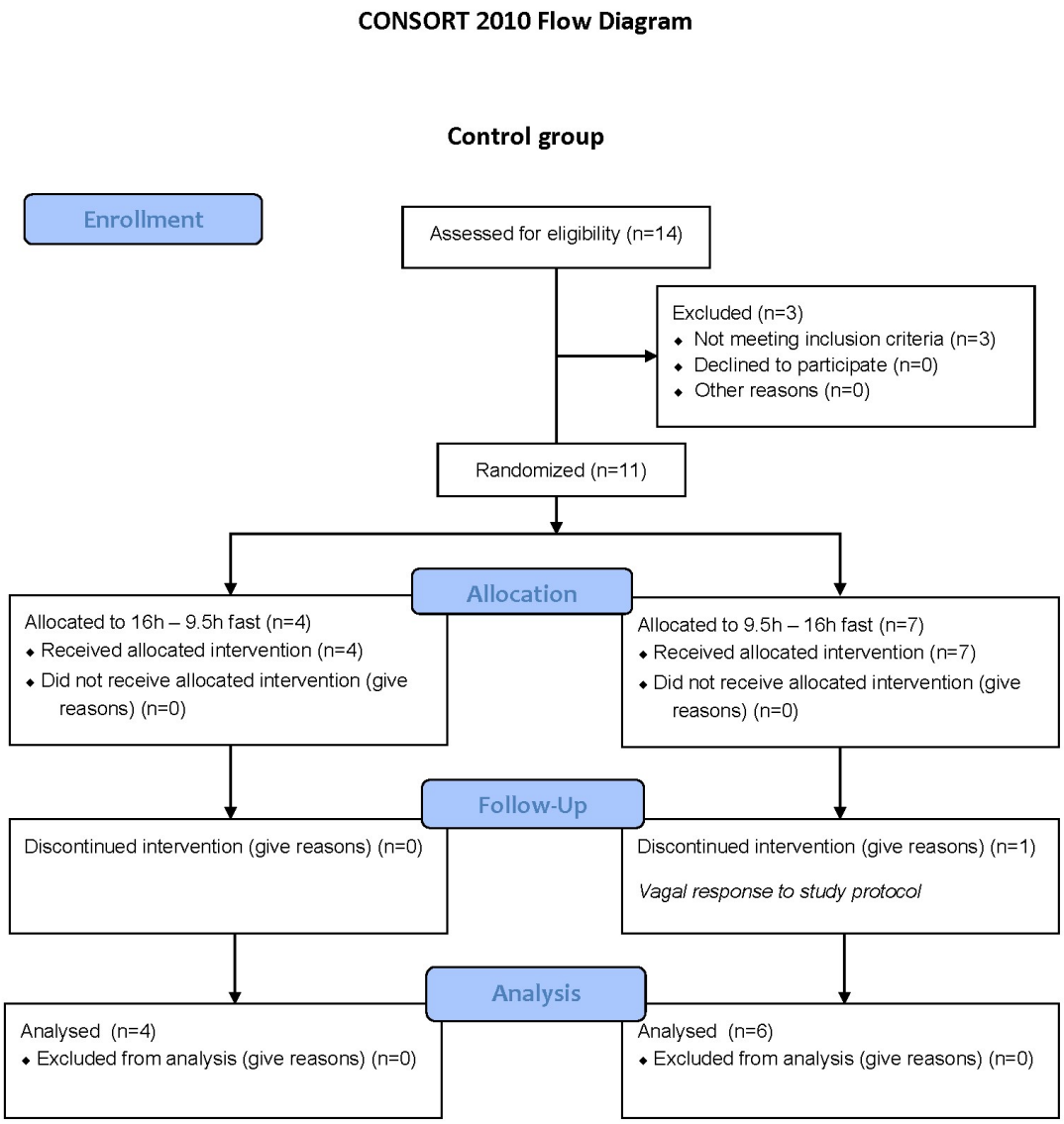
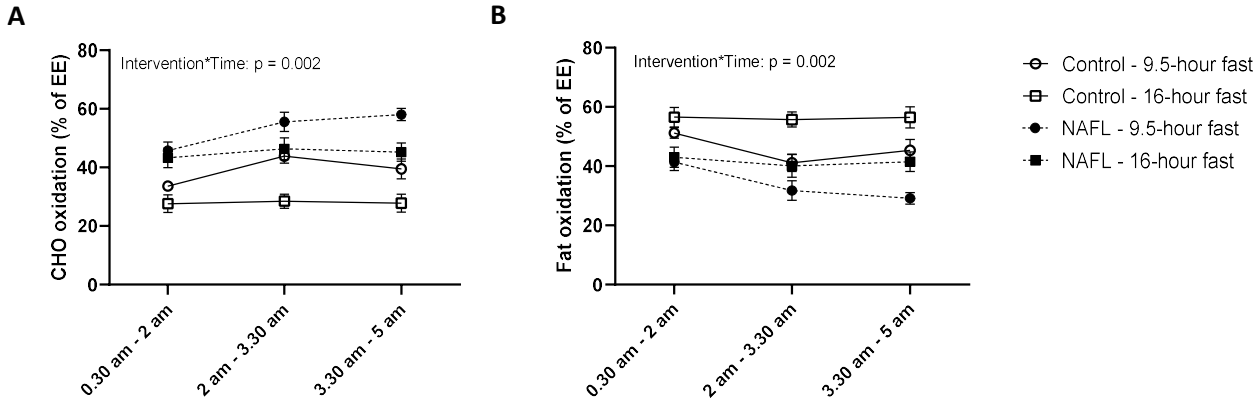
SUPPLEMENTARY MATERIAL FOR CHAPTER 4



SM figure 1 – Participant flowchart of the NAFL group



SM figure 2 – Participant flowchart of the control group



SM Figure 3 - Relative contribution of nocturnal substrate oxidation during 9.5 hours and 16 hours overnight fasting. Relative contribution of carbohydrate oxidation (A) and fat oxidation (B) to energy expenditure over the course of the night in the 9.5-hour and 16-hour fasting condition. For the control group, n = 10; for the NAFL group, n = 11. Data are presented as mean ± SEM.

Chapter 5

Three weeks of time-restricted eating improves glucose homeostasis in adults with type 2 diabetes but does not improve insulin sensitivity: a randomised crossover trial

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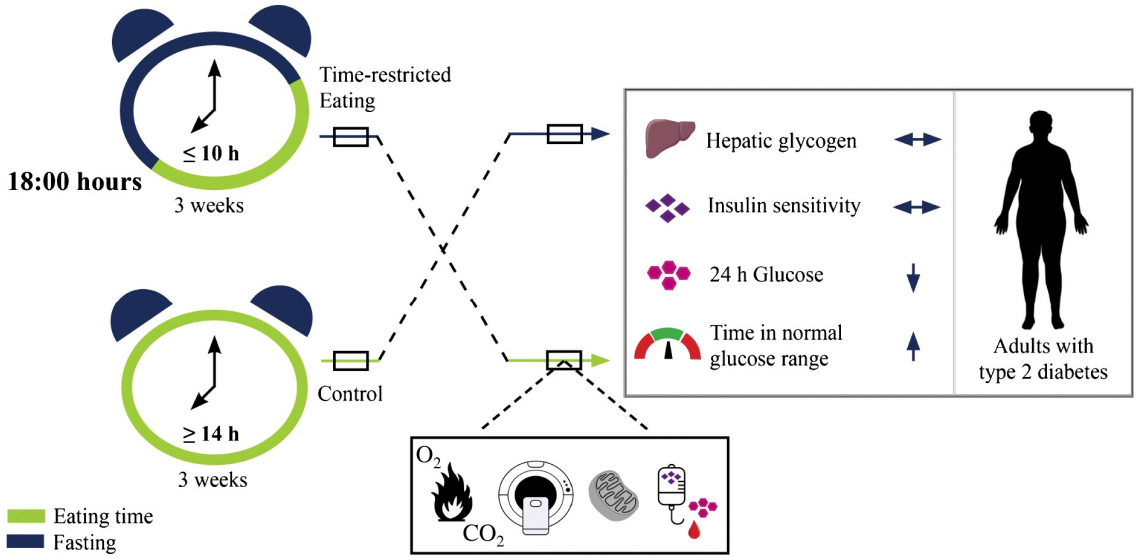

ABSTRACT

Aims/hypothesis Time-restricted eating (TRE) is suggested to improve metabolic health by limiting food intake to a defined time window, thereby prolonging the overnight fast. This prolonged fast is expected to lead to a more pronounced depletion of hepatic glycogen stores overnight and might improve insulin sensitivity due to an increased need to replenish nutrient storage. Previous studies showed beneficial metabolic effects of 6–8 h TRE regimens in healthy, overweight adults under controlled conditions. However, the effects of TRE on glucose homeostasis in individuals with type 2 diabetes are unclear. Here, we extensively investigated the effects of TRE on hepatic glycogen levels and insulin sensitivity in individuals with type 2 diabetes.

Methods Fourteen adults with type 2 diabetes (BMI 30.5±4.2 kg/m^2, HbA_{1c} 46.1±7.2 mmol/mol [6.4±0.7%]) participated in a 3 week TRE (daily food intake within 10 h) vs control (spreading food intake over ≥14 h) regimen in a randomised, crossover trial design. The study was performed at Maastricht University, the Netherlands. Eligibility criteria included diagnosis of type 2 diabetes, intermediate chronotype and absence of medical conditions that could interfere with the study execution and/or outcome. Randomisation was performed by a study-independent investigator, ensuring that an equal amount of participants started with TRE and CON. Due to the nature of the study, neither volunteers nor investigators were blinded to the study interventions. The quality of the data was checked without knowledge on intervention allocation. Hepatic glycogen levels were assessed with ^{13}C-MRS and insulin sensitivity was assessed using a hyperinsulinaemic–euglycaemic two-step clamp. Furthermore, glucose homeostasis was assessed with 24 h continuous glucose monitoring devices. Secondary outcomes included 24 h energy expenditure and substrate oxidation, hepatic lipid content and skeletal muscle mitochondrial capacity.

Results Results are depicted as mean ± SEM. Hepatic glycogen content was similar between TRE and control condition (0.15 ± 0.01 vs 0.15±0.01 AU, p=0.88). M value was not significantly affected by TRE (19.6±1.8 vs 17.7±1.8 µmol kg^{-1} min^{-1} in TRE vs control, respectively, p=0.10). Hepatic and peripheral insulin sensitivity also remained unaffected

by TRE (p=0.67 and p=0.25, respectively). Yet, insulin-induced non-oxidative glucose disposal was increased with TRE (non-oxidative glucose disposal 4.3±1.1 vs 1.5±1.7 µmol kg^{-1} min^{-1}, p=0.04). TRE increased the time spent in the normoglycaemic range (15.1±0.8 vs 12.2±1.1 h per day, p=0.01), and decreased fasting glucose (7.6±0.4 vs 8.6±0.4 mmol/l, p=0.03) and 24 h glucose levels (6.8±0.2 vs 7.6±0.3 mmol/l, p<0.01). Energy expenditure over 24 h was unaffected; nevertheless, TRE decreased 24 h glucose oxidation (260.2±7.6 vs 277.8±10.7 g/day, p=0.04). No adverse events were reported that were related to the interventions.

Conclusions/interpretation We show that a 10 h TRE regimen is a feasible, safe and effective means to improve 24 h glucose homeostasis in free-living adults with type 2 diabetes. However, these changes were not accompanied by changes in insulin sensitivity or hepatic glycogen.

Research in context

What is already known about this subject?

- Most people in Western society spread their food intake over at least 14 h per day, thereby lacking a pronounced fast during the night
- Frequently eating and being active at irregular times, as in night-shift work, has been associated with an increased risk for developing type 2 diabetes
- Limiting food intake to a pre-defined time window (time-restricted eating [TRE]) shows promising beneficial metabolic effects in healthy overweight and obese individuals but mechanisms underlying these benefits are incompletely understood

What is the key question?

- How does following a 3 week 10 h daytime TRE regimen in free-living conditions affect hepatic glycogen and insulin sensitivity in adults with type 2 diabetes?

What are the new findings?

- The 3 week 10 h TRE regimen did not result in alterations in hepatic glycogen and insulin sensitivity as compared with spreading food intake over at least 14 h
- TRE decreased both 24 h and fasting glucose levels and resulted in an increased time spent in normoglycaemia but did not alter energy expenditure or substrate oxidation
- The TRE regimen was safe and feasible to adhere to for adults with type 2 diabetes and, additionally, resulted in weight loss

How might this impact on clinical practice in the foreseeable future?

- Our findings support the use of TRE as an additional strategy for the treatment of type 2 diabetes and provides a foundation to study the long-term effects of TRE in adults with type 2 diabetes

INTRODUCTION

Our modern 24 h society is characterised by ubiquitous food availability, irregular sleep−activity patterns and frequent exposure to artificial light sources. Together, these factors lead to a disrupted day−night rhythm, which contributes to the development of type 2 diabetes (1−3). In Western society, most people tend to spread their daily food intake over a minimum of 14 h (4), likely resulting in the absence of a true, nocturnal fasted state. Restricting food intake to a pre- defined time window (typically ≤12 h), i.e. time-restricted eating (TRE)), restores the cycle of daytime eating and prolonged fasting during the evening and night. Indeed, several studies demonstrated that TRE has promising metabolic effects in overweight or obese individuals, including increased lipid oxidation (5), decreased plasma glucose levels (6, 7) and improved insulin sensitivity (8). While promising, the latter studies applied extremely short eating time windows (e.g. 6–8 h) in highly-controlled settings (5–10), thus hampering implementation into daily life. To date, only Parr et al have successfully explored the potential of TRE in adults with type 2 diabetes using a 9 h TRE regimen (11). However, the effects of TRE on metabolic health remained largely unexplored.

Despite the fact that TRE is sometimes accompanied by (unintended) weight loss (4–6, 9, 10, 12, 13), which inherently improves metabolic health, it has also been reported to improve metabolic health in the absence of weight loss (8), indicating that additional mechanisms underlie the effects of TRE. In this context, individuals with impaired metabolic health display aberrations in rhythmicity of metabolic processes such as glucose homeostasis (14, 15), mitochondrial oxidative capacity (16) and whole-body substrate oxidation (16) compared with the rhythms found in healthy, lean individuals (14, 15, 17). Disruption of circadian rhythmicity is proposed to contribute to the impaired matching of substrate utilisation with substrate availability, which is associated with type 2 diabetes (18). In turn, we hypothesise that these impairments in metabolic rhythmicity are due to a disturbed eating– fasting cycle. Therefore, restricting food intake to daytime and, consequently, extending the period of fasting, may improve metabolic health. More specifically, hepatic glycogen could play a pivotal role in this

process, as it serves as a fuel during the night when glucose levels are low and is replenished during the daytime (19). A decrease in hepatic glycogen triggers the stimulation of fat oxidation and molecular metabolic adaptations that accommodate substrate availability in the fasted state (20), and the need to replenish these stores may improve insulin sensitivity. Hitherto, it is not known whether TRE could result in a more pronounced depletion in hepatic glycogen levels in type 2 diabetes, leading to an improved insulin sensitivity.

The aim of the current study was to examine the effect of limiting food intake to a feasible 10 h daily time frame for 3 weeks in free-living conditions on hepatic glycogen utilisation and insulin sensitivity in adults with type 2 diabetes.

METHODS

This randomised crossover study was conducted between April 2019 and February 2021, after approval of the Ethics Committee of Maastricht University Medical Center (Maastricht, the Netherlands), and conformed with the Declaration of Helsinki (21). The trial was registered at ClinicalTrials.gov (registration no. NCT03992248). All volunteers signed an informed consent form prior to participation. The randomisation procedure is described in the electronic supplementary material (ESM) Methods. The study consisted of two 3-week intervention periods separated by a wash-out period of ≥4 weeks. At the end of each intervention period, main outcomes were measured (ESM Fig. 1) at the Metabolic Research Unit of Maastricht University, the Netherlands. Male and female adults with type 2 diabetes, aged between 50 and 75 years and BMI ≥25 kg/m^2, were eligible for participation. For detailed inclusion and exclusion criteria, see ESM Table 1.

Intervention

During the TRE intervention, volunteers were instructed to consume their habitual diet within a 10 h window during the daytime, with the last meal completed no later than 18:00 hours. Outside this time window, volunteers were only allowed to drink water, plain tea and black coffee. To increase compliance, volunteers were also allowed to drink zero-

energy soft drinks in the evening hours if consumed in moderation. During the control (CON) intervention, volunteers were instructed to spread their habitual diet over at least 14 h per day without additional restraints on the time window of food intake. For both intervention periods, volunteers were instructed to maintain their normal physical activity and sleep patterns and to remain weight stable. Food intake and sleep times were recorded daily using a food and sleep diary. Volunteers based the food intake of their second intervention period on the food and sleep diary filled out during the first period to promote similar dietary quantity and quality in both intervention arms. To optimise compliance, a weekly phone call was scheduled to monitor the volunteers and to provide additional instructions if necessary.

Procedures

At the start of each intervention period, body weight was determined and a continuous glucose monitoring (CGM) device (Freestyle Libre Pro; Abbott, Chicago, USA) was placed on the back of the upper arm to measure interstitial glucose levels every 15 min. On one occasion, between day 7 and 15 of each intervention, fasted hepatic glycogen was measured in the morning at 07:00 hours using ^{13}C-MRS. The day before the MRS measurement, volunteers consumed a standardised meal at home at either 16:40 hours (TRE) or 20:40 hours (CON), ensuring 20 min of meal consumption, so that they were fasted from, respectively, 17:00 hours or 21:00 hours.

On day 19, volunteers arrived at the university at 15:00 hours for measurement of body composition using air displacement plethysmography (BodPod; Cosmed, Rome, Italy), followed by the placement of an i.v. cannula. Afterwards, volunteers entered a respiration chamber for a 36 h measurement of energy expenditure and substrate utilisation using whole-room indirect calorimetry. With TRE, a dinner consisting of 49 per cent of energy (En%) was provided in the respiration chamber at 17:40 hours and volunteers were fasted from 18:00 hours. With CON, a snack of 10 En% was provided at 18:00 hours followed by a 39 En% dinner at 21:40 hours, resulting in a fast from 22:00

hours. Energy content of the meals was based on estimated energy expenditure using the Harris and Benedict equation (22).

On day 20, while in the respiration chamber, a fasted blood sample was obtained at 07:30 hours and 24 h urine was collected for analysis of nitrogen excretion. In both intervention arms, volunteers received standardised meals at fixed times (08:00, 12:00, 15:00 and 18:40 hours) consisting of, respectively, 21 En%, 30 En%, 10 En% and 39 En%. Energy intake was based on sleeping metabolic rate (deter- mined during the night of day 19) multiplied by an activity factor of 1.5. Macronutrient composition of the meals was 56 En% carbohydrates, 30 En% fat and 14 En% protein. Furthermore, volunteers performed low-intensity physical activity at 10:30, 13:00 and 16:00 hours. One bout of activity consisted of 15 min of stepping on an aerobic step and 15 min of standing.

On day 21, after a standardised 11 h fast, volunteers left the respiration chamber at 06:00 hours. Next, a blood sample was taken, followed by measurement of hepatic glycogen and lipid content using ^{13}C-MRS and ^{1}H-MRS, respectively. Subsequently, a muscle biopsy was obtained to assess ex vivo mitochondrial oxidative capacity, after which a hyperinsulinaemic–euglycaemic two-step clamp was started to measure insulin sensitivity. See ESM Methods for detailed descriptions of measurement methods.

Biochemical analyses

Blood samples were used for quantification of metabolites and nitrogen was assessed using 24 h urine samples. See ESM Methods for further details regarding the biochemical analyses.

Data analysis

The statistical packages SPSS Statistics 25 (IBM, New York, USA) and Prism 9 (GraphPad Software, San Diego, USA) were used for statistical analyses. Interventional comparisons are expressed as mean ± SEM. Participant characteristics are expressed as mean ± SD.

Differences between CON and TRE were tested using the paired *t* test, unless specified otherwise. A two-sided $p<0.05$ was considered statistically significant. The power calculation, as well as other calculations made using the measured data, are described in the ESM Methods.

RESULTS

Participant characteristics

A flowchart of participant enrolment is depicted in ESM Fig. 2. Baseline participant characteristics are presented in Table 1. The median Morningness- Eveningness Questionnaire Self-Assessment (MEQ-SA) score amounted to 59.5 (range 41–72). Only one volunteer was identified as an extreme morning type but was included in the study as the intervention did not interfere with his habitual day–night rhythm.

Adherence

Volunteers did not indicate any changes in diabetes medication throughout the study. Volunteers recorded their daily food intake and sleep habits for, on average, 17 days during TRE and 18 days during CON. Based on these data, the eating window averaged 9.1±0.2 h in TRE vs 13.4±0.1 h in CON ($p<0.01$). Sleep–wake patterns were similar in both interventions, with a mean sleep duration of 8.1±0.2 h during TRE and 8.0±0.2 h during CON ($p=0.17$). Body weight at the start of each intervention was comparable between TRE and CON (89.1±3.7 vs 89.2±3.8 kg, respectively, $p=0.62$). Although volunteers were instructed to remain weight stable, a small but significant weight loss occurred in response to TRE (−1.0±0.3 kg, $p<0.01$) but not CON (−0.3±0.3 kg, $p=0.22$). The weight loss with TRE was significantly greater than the weight change observed with CON ($p=0.02$). Body composition determined on day 19 was comparable between TRE and

CON (TRE vs CON: fat mass 37.4±2.7 vs 37.9±2.9 kg, *p*=0.58; and fat-free mass 50.7±2.6 vs 51.0±2.6 kg, *p*=0.60).

Table 1 – Baseline characteristics of participants

Characteristic	Measurement/value
N	14
Sex (F/M)	7/7
Age (yrs)	67.5 ± 5.2
BMI (kg/m^2)	30.5 ± 3.7
Diabetes medication (yes / no)	10/4
Metformin only (n)	7
Metformin + gliclazide (n)	3
Fasting plasma glucose (mmol/l)	7.9 ± 0.4
HbA1c (mmol/mol)	46.1 ± 7.2
HbA1c (%)	6.4 ± 0.7
AST, µkat/l	0.4±0.1
ALT, µkat/l	0.4±0.2
GGT, µkat/l	0.4±0.2
eGFR (ml/min/1.73m2)	79.9 ± 14.5
MEQ-SA (score)	59.1 ± 7.7

Data are shown as mean ± SD, unless stated otherwise.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; MEQ-SA, Morningness-Eveningness Questionnaire Self-Assessment

Hepatic glycogen and lipid content

Approximately half-way through each intervention period, hepatic glycogen levels were assessed in the morning following a 14 h (TRE) and 10 h (CON) night-time fast. Hepatic glycogen did not differ significantly between TRE vs CON (0.16±0.03 vs 0.17±0.02 arbitrary units [AU], respectively, *p*=0.43). At the end of each intervention, hepatic glycogen levels were also assessed after a standardised overnight fast of 11 h for both TRE and CON but did not reveal an altered hepatic glycogen content with TRE compared with CON (0.15±0.01 vs 0.15±0.01 AU, respectively, *p*=0.88). We also assessed hepatic lipid content; neither the amount of lipids nor the composition of the hepatic lipid pool was altered with TRE vs CON (respectively: total lipid content 9.0±2.0 vs 8.6±1.6%, *p*=0.47; polyunsaturated

fatty acids 17.0±1.3 vs 16.2±1.2%, p=0.41; mono-unsaturated fatty acids 40.6±0.9 vs 42.9±1.4%, p=0.19; and saturated fatty acids 42.4±1.2 vs 40.9±1.5%, p=0.41).

Insulin sensitivity and glucose homeostasis

A hyperinsulinaemic–euglycaemic two-step clamp with a glucose tracer and indirect calorimetry was performed to assess insulin sensitivity. No differences in *M* value were found when comparing TRE and CON (19.6±1.8 vs 17.7±1.8 µmol kg^{-1} min^{-1}, respectively, p=0.1). Hepatic insulin sensitivity was not affected by TRE, as exemplified by a similar endogenous glucose production (EGP) with TRE and CON in the fasted state and in the low- and high-insulin- stimulated states (p=0.83, p=0.38 and p=0.30, respectively; Fig. 1a). Suppression of EGP was also similar when comparing TRE with CON upon low- and high-insulin infusion (p=0.67 and p=0.47; Fig. 1a). NEFA suppression upon low insulin exposure was not different between TRE and CON (−365.2±41.6 vs −359.1±43.2 mmol/l, p=0.8). However, absolute levels of NEFAs were lower with TRE during the low- and high-insulin phase (p=0.02 and p=0.04; Fig. 1b), which may hint at an improved adipose tissue insulin sensitivity.

Peripheral insulin-stimulated glucose disposal, reflected by the change in rate of disappearance (R_d) from basal to high insulin, remained unchanged with TRE (p=0.25; Fig. 1c). However, we observed a larger insulin-stimulated non-oxidative glucose disposal (NOGD, difference from baseline to high insulin) with TRE than with CON (4.3±1.1 vs 1.5±1.7 µmol kg^{-1} min^{-1}, respectively, p=0.04; Fig. 1d) reflecting an increased ability to form glycogen. Conversely, insulin-stimulated carbohydrate oxidation from basal to high insulin appeared to be lower with TRE than with CON (4.7±0.9 vs 6.2±0.9 µmol kg^{-1} min^{-1}, respectively) but this difference was not statistically significant (p=0.07). Consistently, insulin-induced suppression of fat oxidation from basal to high-insulin was lower with TRE than with CON (−1.3±0.3 vs −1.8±0.2 µmol kg^{-1} min^{-1}, p=0.04; Fig. 1e). Energy expenditure did not differ between TRE and CON during the basal, low-insulin and high-insulin phase of the clamp. These results indicate that while peripheral insulin sensitivity is unchanged with TRE, glucose uptake is more directed towards storage compared with

oxidation. Both hepatic and peripheral insulin sensitivity, as well as levels of hepatic glycogen, were additionally analysed in volunteers who only used metformin as diabetes treatment (*n*=7) and this did not alter the outcomes.

To examine the effect of TRE on glucose homeostasis, CGM data from the last 4 days in the free-living situation (days 15–18) were analysed for both interventions. Four volunteers presented incomplete CGM data due to technical issues, hence statistics were performed on CGM data from ten volunteers. Mean 24 h glucose levels were lower in TRE compared with CON (6.8±0.2 vs 7.6±0.3 mmol/l, $p<0.01$; Fig. 2a–f). Nocturnal glucose levels were consistently lower in TRE vs CON (Fig. 2a–d). Furthermore, volunteers spent more time in the normal glucose range upon TRE compared with CON (15.1±0.8 vs 12.2±1.1 h per day, $p=0.01$; Fig. 2f). Concomitantly, time spent in the high glucose range was less in TRE compared with CON (5.5±0.5 vs 7.5 0.7 h per day, $p=0.02$) whereas no differences between eating regimens were found for time spent in hyperglycaemia (2.3±0.4 vs 3.7±0.8 h per day, $p=0.24$), time spent in the low glucose range (0.5±0.1 vs 0.4±0.1 h per day, $p=1.00$) or time spent in hypoglycaemia (0.7±0.3 vs 0.1±0.0 h per day, $p=0.48$).

Additionally, fasting plasma metabolites were assessed on day 20 and day 21 of each intervention. On day 20, blood samples were taken after a 10 h (CON) or 14 h (TRE) overnight fast. Plasma glucose on day 20 was lower after TRE (7.6±0.4 vs 8.6±0.4 mmol/l, respectively, $p=0.03$) whereas plasma insulin, triglycerides (TG), and NEFA levels were comparable between conditions (Table 2). On day 21, when overnight fasting time was similar for both interventions (11 h), plasma glucose levels remained lower in TRE than in CON (8.0±0.3 vs 8.9±0.5 mmol/l, respectively, $p=0.04$), whereas no differences were detected in plasma insulin, TG and NEFA levels (Table 2).

Twenty-four-hour energy and substrate metabolism

On day 19, volunteers resided in a respiration chamber for 36 h for measurement of energy expenditure and substrate oxidation. Twenty-four-hour energy expenditure was

similar for TRE and CON (9.57±0.22 vs 9.68±0.29 MJ/day, respectively, *p*=0.22; Fig. 3a), as was the 24 h respiratory exchange ratio (RER) (0.86±0.01 vs 0.86±0.01, respectively, *p*=0.13). Nonetheless, 24 h carbohydrate oxidation was lower in TRE vs CON (260.2±7.6 vs 277.8±10.7 g/day, respectively, *p*=0.04; Fig. 3b), whereas 24 h fat oxidation (91.9±6.6 vs 93.5±5.5 g/day, respectively, *p*=0.72; Fig. 3c) was unaffected. Twenty-four-hour protein oxidation seemed higher upon TRE but the difference did not reach statistical significance (72.8 ±7.2 vs 58.5±5.4 g/day, respectively, *p*=0.18; Fig. 3d). Sleeping metabolic rate appeared to be lower with TRE compared with CON (4.66±0.14 vs 4.77±0.18 kJ/min, respectively), although this decrease was not statistically significant (*p*=0.05; Fig. 3e). There was no change in carbohydrate or fat oxidation during sleep in response to TRE vs CON (RER 0.84±0.01 vs 0.84±0.01, p=0.50; Fig. 3f). On day 21, muscle biopsies were obtained to assess ex vivo mitochondrial oxidative capacity by means of high-resolution respirometry. In total, paired biopsies from 13 out of 14 volunteers were analysed. Mitochondrial respiration did not differ between TRE and CON (Table 3).

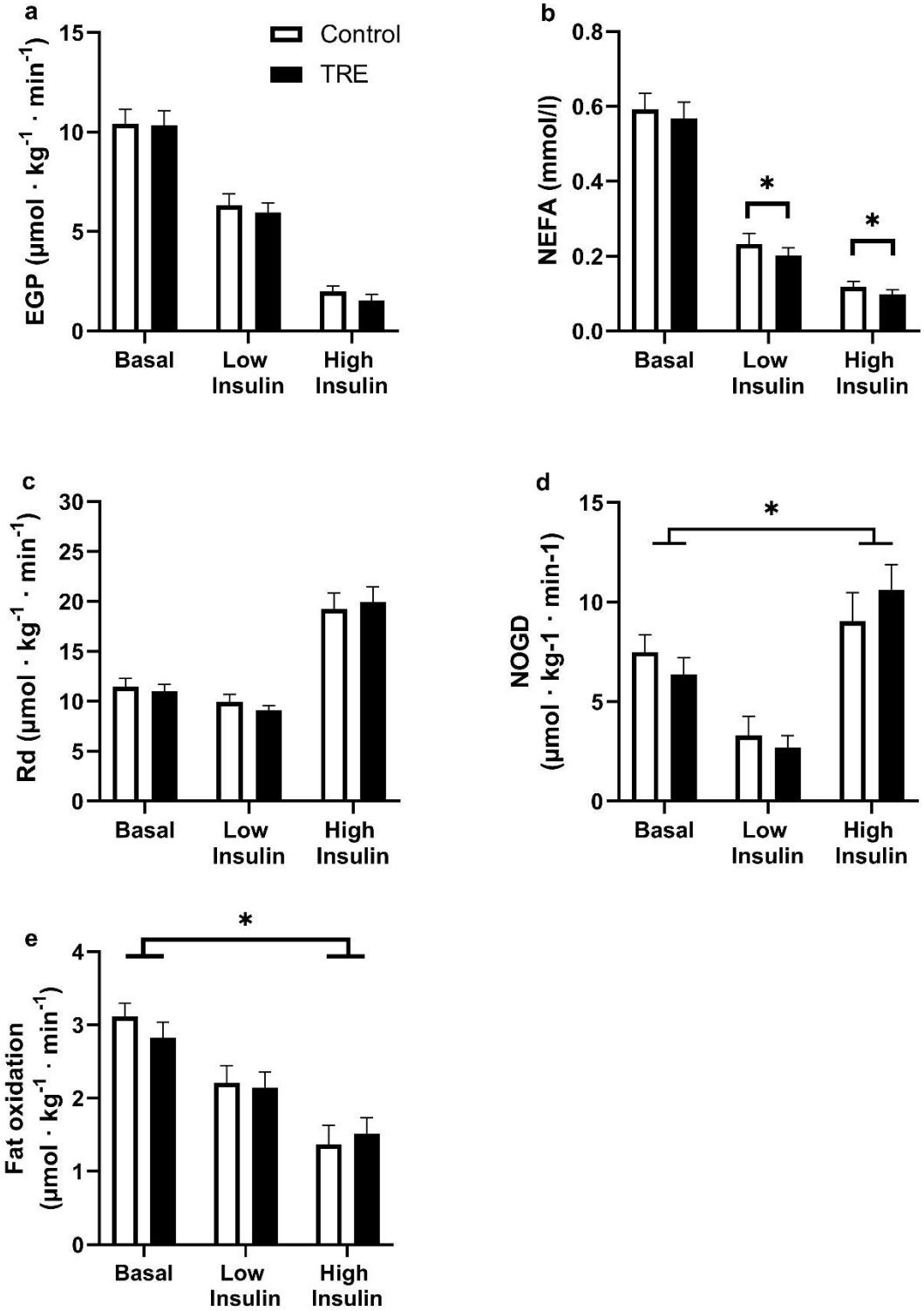

Fig. 1 Effect of TRE on EGP (a), plasma NEFA (b), R_d (c), NOGD (d) and fat oxidation (e) measured during a hyperinsulinaemic– euglycaemic two-step clamp (n=14). *p<0.05 (data were analysed with paired t tests). R_d, Rate of disappearance

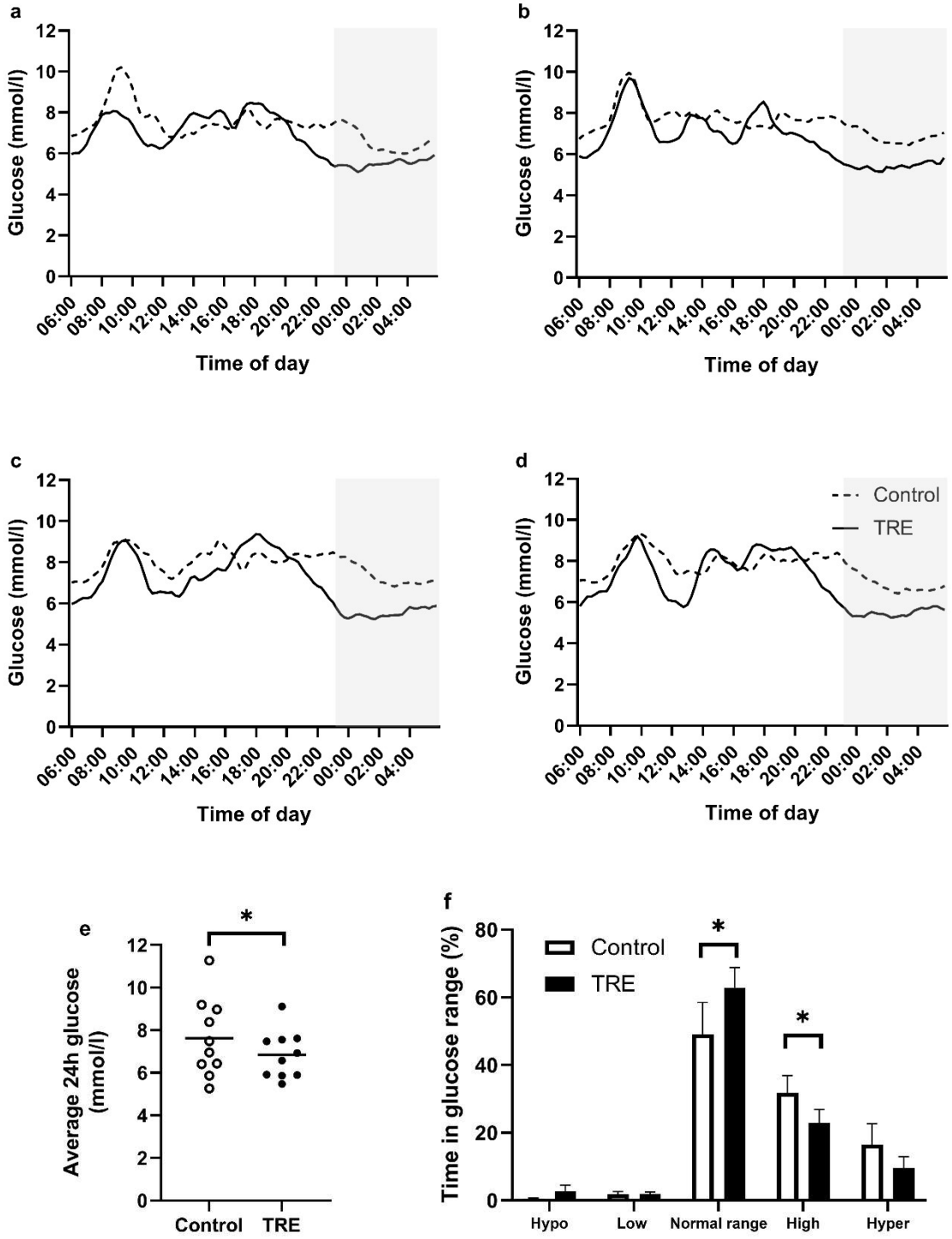

Fig. 2 (a–d) Twenty-four-hour glucose levels on days 15 (a), 16 (b), 17 (c) and 18 (d) during TRE or CON (*n*=10). Mean 24 h glucose from day 15 to day 18 (*n*=10) analysed using a paired *t* test (e). Time spent in glucose range during days 15–18 (*n*=10) analysed using Wilcoxon tests with Bonferroni correction (f) *$p<0.05$. Hypo, hypoglycaemia defined as glucose levels <4.0 mmol/l; Low, low glucose levels defined as glucose levels 4.0–4.3 mmol/l; Normal range, glucose levels within

the normal range defined as 4.4–7.2 mmol/l; High, high glucose levels defined as glucose levels 7.3–9.9 mmol/l; Hyper, hyperglycaemia defined as glucose levels >10 mmol/l

Table 2 Blood plasma biochemistry

Metabolite	CON	TRE	*p* value
Day 20 (*n*=13)			
Triglycerides, mmol/l	2.1±0.3	1.9±0.2	0.30
NEFA, mmol/l	0.529±0.038	0.489±0.035	0.39
Glucose, mmol/l[a]	8.6±0.4	7.6±0.4	0.03
Insulin, pmol/l	111.1±20.8	104.2±13.9	0.27
Day 21 (*n*=14)			
Triglycerides, mmol/l	2.1±0.3	2.2±0.2	0.66
NEFA, mmol/l	0.601±0.070	0.542±0.064	0.30
Glucose, mmol/l[b]	8.9±0.5	8.0±0.3	0.04
Insulin, pmol/l	97.2±13.9	111.1±20.8	0.16

Data are shown as mean ± SEM

[a] Fasted blood values with fasting time 10 h for CON and 14 h for TRE

[b] Fasted blood values with fasting time 11 h for both CON and TRE

Table 3 Mitochondrial oxidative capacity

Respiration state	CON	TRE	*p* value
State 2[a]			
M, pmol mg^{-1} s^{-1}	5.0±0.4	5.5±0.6	0.49
MO, pmol mg^{-1} s^{-1}	6.6±0.4	6.8±0.5	0.93
MG, pmol mg^{-1} s^{-1}	7.1±0.4	6.9±0.4	0.54
State 3[b]			
MO, pmol mg^{-1} s^{-1}	28.3±2.0	29.1±1.4	0.91
MG, pmol mg^{-1} s^{-1}	31.2±1.9	32.6±1.8	0.82
MOG, pmol mg^{-1} s^{-1}	37.0±2.3	37.8±1.9	0.99
MOGS, pmol mg^{-1} s^{-1}	55.7±3.3	57.6±2.7	0.80
State U[c]			
MGS, pmol mg^{-1} s^{-1}	58.0±3.2	59.9±2.9	0.91
FCCP, pmol mg^{-1} s^{-1}	67.8±4.7	66.6±3.5	0.50
State 4o[d]			
Oligomycin, pmol mg^{-1} s^{-1}	17.6±1.2	18.1±1.2	0.85

Data presented as mean ± SEM, *n*=13

[a] State 2, respiration in presence of substrates alone

[b] State 3, ADP-stimulated respiration

[c] State U, maximal respiration in response to an uncoupling agent

[d] State 4o, mitochondrial proton leak measured by blocking ATP synthase

FCCP, trifluoro-methoxy carbonyl cyanide-4 phenylhydrazone; G, gluta- mate; M, malate; O, octanoyl-carnitine; S, succinate

DISCUSSION

TRE is a novel strategy to improve metabolic health and has been proposed to counteract the detrimental effects of eating throughout the day by limiting food intake to daytime hours. To date, only a few studies have examined the metabolic effects of TRE in adults with type 2 diabetes. Here, we tested whether restricting energy intake to a feasible, 10 h time frame for 3 weeks would lower hepatic glycogen levels and improve insulin sensitivity in overweight/obese adults with type 2 diabetes. Additionally, we explored the effects of TRE on glucose homeostasis, 24 h energy metabolism and mitochondrial function.

We hypothesised that the 10 h TRE regimen, with the latest food intake at 18:00 hours, would result in a more pronounced fasting state, especially during the night. During the night, the liver is crucial to the regulation of blood glucose through the processes of gluconeogenesis and glycogenolysis and it has been shown that these processes are elevated in type 2 diabetes (23, 24). Therefore, we hypothesised that hepatic glycogen would be lower after TRE and would be associated with an improved insulin sensitivity.

In contrast to our hypothesis, hepatic glycogenolysis appeared to be unaffected by TRE since there was no change in glycogen content after a standardised 11 h fast at the end of the intervention. Neither was there a change after a 14 h (TRE) vs 10 h (CON) overnight fast half-way through the intervention. In addition, EGP suppression during the low-insulin phase of the hyperglycaemic–euglycaemic clamp (reflecting hepatic insulin sensitivity) did not differ between TRE and CON. A limitation of our approach is that we did not measure hepatic glycogen dynamics during the night. Such measurements may be important, as our clamp results showed an increase in NOGD upon high-insulin stimulation with TRE, suggesting an increased glycogen storage. These results could suggest small changes in hepatic glycogen turnover; alternatively, muscle glycogen levels may play a role in explaining our clamp results as the muscle accounts for most of the glycogen synthesis upon high-insulin stimulation in healthy individuals. Interestingly, type 2 diabetes is characterised by an impaired insulin-stimulated glycogen storage (25). Thus, an improvement in NOGD due to TRE may help to regulate 24 h and postprandial glucose levels. Indeed, 24 h glucose levels were significantly improved after TRE.

We did not observe an effect of TRE on insulin sensitivity. A previous controlled randomised crossover study by Sutton et al did show an improved insulin sensitivity with TRE (8). Thus, men with prediabetes followed a 5 week 6 h early TRE regimen, whereby the last meal was consumed before 15:00 hours. The differences in results may be explained by the shorter eating window and earlier consumption of the last meal (15:00 vs 18:00 hours), creating a longer period of fasting. Here, we chose a 10 h TRE, which we believe would be feasible to incorporate into the work and family life of most adults with type 2 diabetes; future studies will be needed to reveal whether the duration of the fasting period is indeed crucial in determining positive effects on insulin sensitivity.

Despite the lack of changes in hepatic glycogen and insulin sensitivity, we did find that our 10 h TRE protocol decreased 24 h glucose levels in individuals with type 2 diabetes, primarily driven by decreased nocturnal glucose levels. Notably, TRE also lowered overnight fasting glucose, increased the time spent in the normal glucose range and decreased time spent in the high glucose range, all of which are clinically relevant variables in type 2 diabetes. Importantly, morning fasting glucose levels were consistently lower with TRE than with CON, even when the fasting duration prior to the blood draw was similar between the two interventions. This may indicate lasting changes in nocturnal glucose homeostasis. Additionally, we found that time spent in hypoglycaemia was not significantly increased upon TRE and no serious adverse events were reported resulting from TRE, thereby underscoring that a ~10 h eating window is a safe and effective lifestyle intervention for adults with type 2 diabetes.

Mechanisms underlying the improvement in glucose homeostasis upon TRE remain unclear. Our results show that TRE did not improve peripheral and hepatic insulin sensitivity, skeletal muscle mitochondrial function, energy metabolism or hepatic lipid content, all of which are known to be affected in type 2 diabetes mellitus (25–29). Under high- insulin conditions during the clamp, we observed a larger reliance on fatty acid oxidation accompanied by higher NEFA levels and lower glucose oxidation. Lower glucose oxidation was also observed when measured over 24 h. Although not statistically significant, the mean of 24 h protein oxidation was higher with TRE, possibly reflecting a

more pronounced fasting state and a drive towards a higher rate of amino-acid-driven gluconeogenesis. A previous study by Lundell et al indeed suggested that TRE could affect protein metabolism to cope with the extended period of fasting (30). However, the exact mechanisms and implications of these effects require further investigation, and it would be interesting to investigate nocturnal glucose metabolism in more detail. The improvement in glucose homeostasis may also partially be explained by the weight loss induced by TRE, which has also been reported previously (4–6, 9, 10, 13, 31). It should be noted, however, that the body weight loss was rather small (~0.7 kg compared with CON after 3 weeks of intervention) which makes it less likely to completely explain the differences in glucose homeostasis.

A limitation of the current study is the relatively heterogeneous study population consisting of adults with and without use of glucose-lowering medication. Use of medication might have resulted in TRE having less effect, as the medication may be targeting the same metabolic pathways. Only recruiting volunteers not receiving medication would have prevented this issue but would have made the results less applicable to the general type 2 diabetes population. Another limitation of our study is the relatively short duration of 3 weeks. This duration was chosen as the aim of this study was to assess whether TRE would result in metabolic improvements in type 2 diabetes and to explore potential mechanisms underlying these changes. In our experience, human interventions of 3 weeks are able to affect the outcome variables investigated in our study. Since our TRE protocol was feasible and safe, and resulted in improved 24 h glucose levels, it would be interesting to examine the impact of 10 h TRE on glucose homeostasis and insulin sensitivity in type 2 diabetes in the long term to address the clinical relevance of TRE.

In conclusion, we show that a daytime 10 h TRE regimen for 3 weeks decreases glucose levels and prolongs the time spent in normoglycaemia in adults with type 2 diabetes as compared with spreading daily food intake over at least 14h. These improvements were not mediated by changes in hepatic glycogen, insulin sensitivity, mitochondrial function or 24 h substrate oxidation. These data highlight the potential benefits of TRE in type 2 diabetes.

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AUTHOR CONTRIBUTIONS

CA, CEF, JH and PS designed the experiments. CA, CEF, AV, KHMR, SMMvB, EM-K, NJC and BH performed the measurements. CA, AV, VBS-H, JM, JH and PS were involved in data analysis. CA, JH and PS drafted the manuscript. All authors reviewed and approved the final version of the manuscript. PS is the guarantor of this work and, as such, has full access to the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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SUPPLEMENTARY MATERIAL FOR CHAPTER 5

METHODS

Research Design and Methods

Prior to the study, a randomisation schedule was constructed by a study-independent researcher using the website randomizer.org. Intervention sequences (TRE:CON or CON:TRE) were constructed using blocks of equal size which ensured that an equal number of patients started with CON as with TRE. Due to the nature of the study, neither coordinating investigator nor patients were blinded to the intervention. When volunteers were found eligible to participate, study enrolment and allocation to either TRE and CON (based on the randomisation schedule) was performed by the coordinating investigator.

Procedures

A 3.0 T clinical MRI scanner (Achieva Philips Healthcare, Best, the Netherlands) was used to perform the measurements of hepatic glycogen and hepatic lipid content. Glycogen was measured using ^{13}C-MRS with a dedicated ^{13}C / ^{1}H coil and the volunteer lying in prone position. Power settings were calibrated to achieve a 90-degree pulse in the liver (at 8 cm from the coil) and spectra were acquired without 1H-decoupling. The area under the curve of the glycogen doublet at 100.5 ppm was determined using MATLAB and phantom-based sensitivity maps and MRI image segmentation were used to correct for coil sensitivity in the liver area. For hepatic lipid content, volunteers were positioned in the supine position and a volume of interest was selected within the right lobe of the liver to acquire ^{1}H-MRS spectra using STEAM (TE: 20 ms, TR: 4500ms, number of averages: 128) [1]. Volunteers were asked to breathe in a rhythm to prevent motion artefacts. The water signal was suppressed by VAPOR water suppression. A spectrum without water suppression was also acquired to quantify the water signal and the ratio of lipid over the sum of lipid and water was determined. From this, absolute values of fat percentage were deduced [2] and

hepatic lipid content is given as weight/weight percentage. From the lipid spectrum, the relative contribution of saturated, monounsaturated and polyunsaturated fatty acids were determined according to Roumans et al [1].

To measure mitochondrial oxidative capacity, permeabilized muscle fibres were prepared freshly directly after the muscle biopsy as described previously [4]. Subsequently, the permeabilized muscle fibres (~2.5 mg wet weight) were analysed for mitochondrial function using an oxygraph (OROBOROS Instruments, Innsbruck, Austria). To prevent oxygen limitation, the respiration chambers were hyper-oxygenated up to ~400 µmol L^{-1} O2. Subsequently, two different multi-substrate/inhibition protocols were used in which substrates (malate, octanoyl-carnitine, glutamate, succinate) and other compounds (ADP, oligomycin, FCCP) were added consecutively at saturating concentrations to characterize mitochondrial capacity, as described previously [5]. Measurements were performed in quadruplicate and cytochrome c was added upon maximal coupled respiration (state 3) to assess mitochondrial membrane integrity. If oxygen consumption increased >15% after cytochrome c addition, that particular measurement was excluded from analysis.

The hyperinsulinemic-euglycemic two-step clamp was performed to measure insulin sensitivity, as described previously [3]. Briefly, the clamp started with 120 minutes of primed- continuous infusion of D-[6,6-^{2}H2] glucose to determine baseline endogenous glucose production (EGP), glucose appearance (Ra) and glucose disposal (Rd). Afterwards, insulin was infused at 10 mU · m^2 · min^{-1} to assess hepatic insulin sensitivity reflected by suppression of EGP. After 3 h, insulin infusion was increased to 40 mU · m^2 · min^{-1} to measure muscle insulin sensitivity. Arterialized blood was drawn every 5-10 minutes to assess glucose levels and glucose (20%) was co-infused to maintain glucose levels at ~5 mmol/l. Energy expenditure and substrate utilization was measured during the last 30 min of every steady- state period (basal, low insulin and high insulin) using indirect calorimetry (Omnical; Maastricht Instruments, Maastricht, the Netherlands).

Biochemical analyses

The ABX Pentra C400 (Horiba, Montpellier, France) was used to enzymatically quantify triglycerides (Sigma, St Louis, USA), free fatty acids (FFAs) (Wako, Neuss, Germany) and glucose concentrations (Horiba, Montpellier, France) in EDTA plasma. Insulin levels were determined using enzyme-linked immunoassay in EDTA plasma (Crystal Chem Inc, Illinois, USA). Nitrogen was assessed in 24-hour urine samples using the Vario Max (Elementar Analysensysteme GmbH, Langenselbold, Germany). Samples from volunteers were analysed in the same run for both interventions.

Data analysis

For determination of glucose homeostasis, continuous glucose monitor data was obtained in the free-living situation of both TRE and CON. Data from the last 4 days (day 15 – 18) was combined to account for day-to-day specific effects on glucose excursions. This continuous data was divided into categories defined by the American Diabetes Association [6, 7]: hypoglycaemia < 4.0 mmol/l, low blood glucose 4.0 – 4.3 mmol/l, normal range 4.4 – 7.2 mmol/l, high blood glucose 7.3 – 9.9 mmol/l, hyperglycaemia > 10.0 mmol/l. Results were reported as percentage of time spent in the respective categories and differences between TRE and CON were tested using multiple Wilcoxon signed rank tests with a Bonferroni correction.

Sleeping metabolic rate was defined as the lowest 3 h of nocturnal energy expenditure during the first night in the respiration chamber and calculated with the Weir equation [8].

Twenty-four-hour energy expenditure and -substrate utilization were calculated using the equation from Brouwer et al. [9] with data obtained from the last 24 h of the respiration chamber measurement, including 24-hour urine collection to determine protein oxidation. From the indirect calorimetry data collected during the clamp, carbohydrate- and fat oxidation were calculated using the Brouwer equation [9] with protein oxidation

being estimated as 12.4% of energy expenditure. In addition, Steele's singe pool non-steady state equations were used to calculate Ra and Rd [10].

Sample size was determined based on the variability in glycogen content measured in the fasted state with ^{13}C-MRS, which amounted 7 – 11% in previous studies [11, 12]. For a more conservative estimation of the standard deviation, 11% was used in our calculation. The following equation was used for sample size calculation: $N = \sigma^2/\Delta\mu^2 * (Z0.8 + Z0.975)^2$ with Z0.8

= 0.842, Z0.975 = 1.960, σ^2 = 11%, and $\Delta\mu^2$ = 10%. Filling out this equation indicated that we needed to included 10 volunteers in our study. However, since the variation in hepatic glycogen in response to a time restricted eating regime was not investigated at the time of the calculation and might be greater, we decided to include 14 volunteers in our final data analyses.

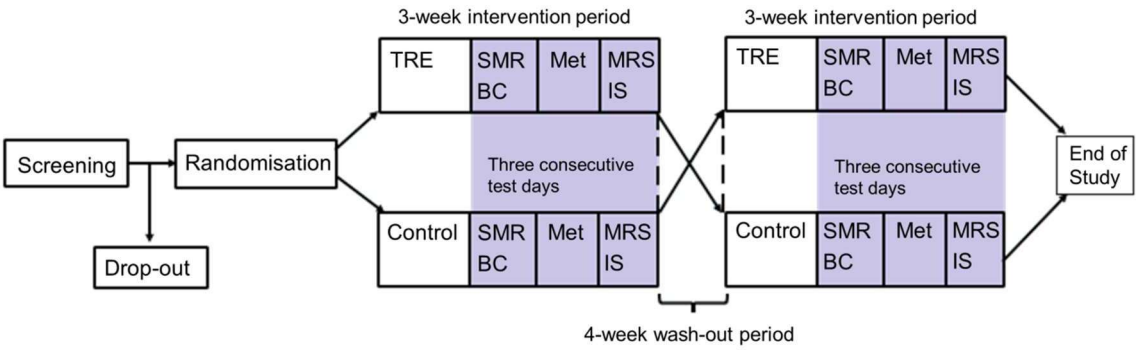
TABLES

ESM Table 1: Inclusion criteria of the trial

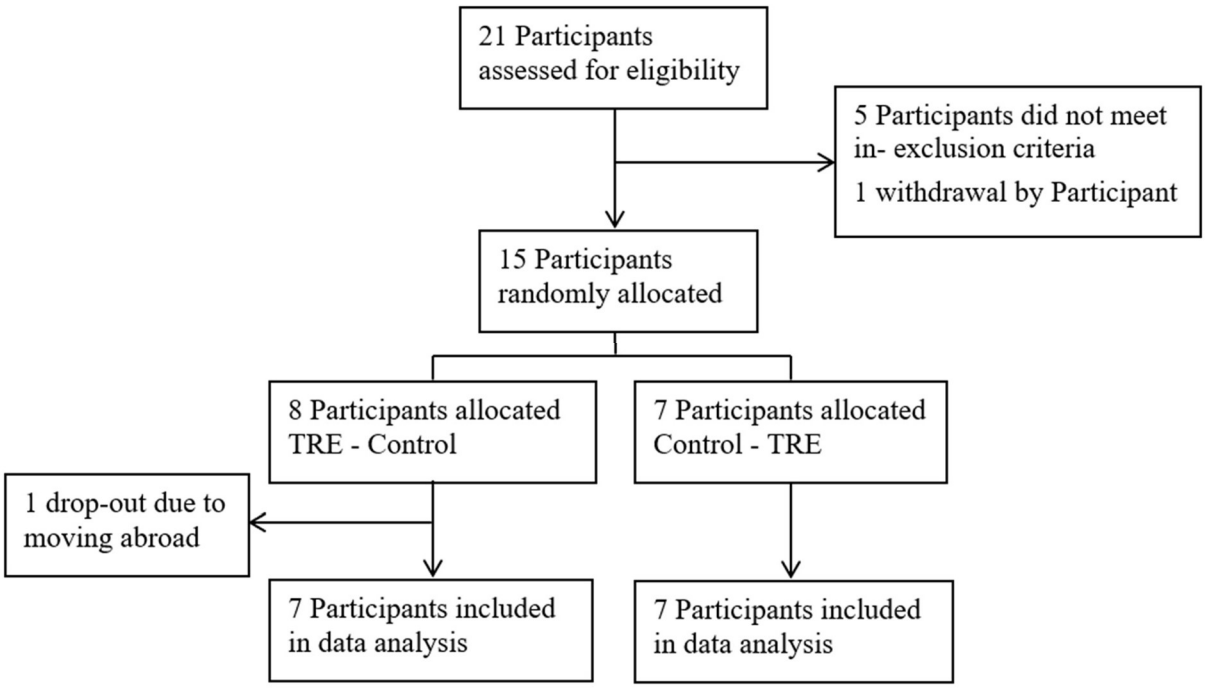
Inclusion criteria	
1	Patients are able to provide signed and dated written informed consent prior to any study specific procedures
2	Caucasian
3	Non-insulin treated type 2 diabetes
4	Women are post-menopausal (defined as at least 1 year cessation of menses)
5	Age: 50 – 75 years
6	BMI ≥ 25 kg/m^2
7	Regular sleeping time (normally 7 – 9h daily)
8	Habitual sleeping time 11 PM ± 2 h
Exclusion criteria	
1	Not being able to adhere to a restricted eating schedule
2	Uncontrolled hypertension
3	Active cardiovascular disease
4	Insulin therapy
5	Use of sodium-glucose costransporter-2 inhibitors
6	Engagement in programmed exercise for >3h per week
7	Extreme early bird or extreme night person (score ≤30 or ≥70 on morningness- eveningness questionnaire – self assessment questionnaire)

8	Heavily varying sleep-wake rhythm
9	Shiftwork during last 3 months
10	Smoking
11	Contra-indication to Magnetic Resonance Imaging (MRI) scanning
12	Blood donation during intervention or less than three months before the start of intervention
13	Not willing to be informed about unexpected medical findings during the screening/study, or not willing to have the attending general practitioner informed about study (findings)
14	Unstable body weight (weight gain or loss > 3 kg during 3 months prior to study onset)
15	Significant food allergies/intolerance (seriously hampering study meals)
16	Participation in another biomedical study within 1 month before the first study visit, which would possibly hamper our study results
17	Another medical condition that will preclude the safe performance of the measurements as judged by the medical doctor

FIGURES



ESM Figure 1 – Overview of the study design of the time restricted eating study. TRE: time restricted eating, SMR: sleeping metabolic rate, BC: body composition, Met: 24-hour energy metabolism, MRS: magnetic resonance spectroscopy, IS: insulin sensitivity



ESM Figure 2 – Flowchart of the time restricted eating study

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Chapter 6

Effect of the SGLT2 inhibitor dapagliflozin on substrate metabolism in humans with prediabetes: a randomized, double-blind crossover trial

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ABSTRACT

Aims/hypothesis Sodium-glucose cotransporter 2 inhibitor (SGLT2i) treatment in type 2 diabetes mellitus patients results in glucosuria, causing an energy loss, and triggers beneficial metabolic adaptations. It is so far unknown if SGLT2i exerts beneficial metabolic effects in prediabetic insulin resistant individuals, yet this is of interest since SGLT2is also reduce the risk for progression of heart failure and chronic kidney disease in patients without diabetes.

Methods Fourteen prediabetic insulin resistant individuals (BMI: 30.3±2.1 kg/m^2; age: 66.3±6.2 years) underwent 2-weeks of treatment with dapagliflozin (10mg/day) or placebo in a randomized, placebo-controlled, cross-over design. Outcome parameters include 24-hour and nocturnal substrate oxidation, and twenty-four-hour blood substrate and insulin levels. Hepatic glycogen and lipid content/composition were measured by MRS. Muscle biopsies were taken to measure mitochondrial oxidative capacity and glycogen and lipid content.

Results Dapagliflozin treatment resulted in a urinary glucose excretion of 36 gram/24-hour, leading to a negative energy and fat balance. Dapagliflozin treatment resulted in a higher 24-hour and nocturnal fat oxidation (p=0.043 and p=0.039, respectively), and a lower 24-hour carbohydrate oxidation (p=0.048). Twenty-four-hour plasma glucose levels were lower (AUC; p=0.016), while 24-hour free fatty acids and nocturnal β-hydroxybutyrate levels were higher (AUC; p=0.002 and p=0.012, respectively) after dapagliflozin compared to placebo. Maximal mitochondrial oxidative capacity was higher after dapagliflozin treatment (dapagliflozin: 87.6±5.4, placebo: 78.1±5.5 pmol/mg/s, p=0.007). Hepatic glycogen and lipid content were not significantly changed by dapagliflozin compared to placebo. However, muscle glycogen levels were numerically higher in the afternoon in individuals on placebo (morning: 332.9±27.9, afternoon: 368.8±13.1 nmol/mg), while numerically lower in the afternoon on dapagliflozin treatment (morning: 371.7±22.8, afternoon: 340.5±24.3 nmol/mg).

Conclusions/interpretation Dapagliflozin treatment of prediabetic insulin resistant individuals for 14 days resulted in significant metabolic adaptations in whole-body and skeletal muscle substrate metabolism despite being weight neutral. Dapagliflozin improved fat oxidation and *ex vivo* skeletal muscle mitochondrial oxidative capacity, mimicking the effects of calorie restriction.

INTRODUCTION

The sodium-glucose cotransporter 2 (SGLT2) inhibitor (SGLT2i) dapagliflozin is approved for treatment of type 2 diabetes mellitus (T2DM) as well as heart failure and chronic kidney disease irrespective of T2DM (1-3). SGLT2i treatment results in the excretion of approximately 60 – 90 grams of glucose per day in patients with T2DM. This glucosuria has been shown to induce a clinically relevant reduction in HbA1c and fasting glucose in patients with T2DM (4, 5). In addition, SGLT2i reduces body weight and blood pressure (5-7), and restores water and sodium balance (8, 9). We (10) and others (11-13) have shown that SGLT2i treatment reduces carbohydrate oxidation and increases fat oxidation in patients with T2DM. Additionally, SGLT2i decreases diurnal plasma insulin levels and increases plasma free fatty acid, β-hydroxybutyrate, and glucagon levels (10, 14), and reduces hepatic lipid content in patients with T2DM (10, 15-17).

The loss of urinary glucose by SGLT2i also leads to a compensatory increase in endogenous glucose production (EGP) in the overnight fasted state (10-12, 18, 19). We hypothesized that – next to gluconeogenesis - increased glycogenolysis is responsible for the higher EGP. Such increased glycogenolysis could lead to a reduction of hepatic glycogen levels overnight, which could explain beneficial metabolic adaptations such as improvements in mitochondrial function and increased fat oxidation (20, 21), similar to those observed after caloric restriction (10).

Not only T2DM patients, but also prediabetic insulin resistant individuals are characterized by metabolic disturbances (22, 23). Therefore, investigating potential effects of SGLT2i in prediabetic insulin resistant individuals on energy and substrate metabolism is of interest since restoring the 24-hour energy metabolism may be one of the mechanisms of SGLT2i contributing to reduced progression of chronic kidney

disease and heart failure in patients without T2DM (1-3), who are, however, insulin resistant (24, 25).

Therefore, for the first time, we have investigated if SGLT2i treatment improves 24-hour energy- and substrate- metabolism in prediabetic insulin resistant individuals and additionally, examine the subsequent SGLT2i effects on skeletal muscle mitochondrial oxidative capacity as a potential underlying mechanism of benefit. Furthermore, we test the hypothesis that the beneficial effects of SGLT2i on metabolic health include a reduction in overnight fasted hepatic glycogen levels as compensation for the urinary glucose loss.

RESEARCH DESIGN AND METHODS

Study design and participants

The study had a double-blinded, randomized, placebo-controlled, cross-over design and was conducted at Maastricht University, the Netherlands, between April 2019 and July 2021. Due to the COVID-19 pandemic, the study completion was delayed. The Ethics Committee of Maastricht University Medical Center approved the study, which was registered at clinicaltrials.gov (NCT03721874) and conducted conform the declaration of Helsinki (26). Male and female individuals between 40 – 75 years and BMI of 27 – 38 kg/m^2 without T2DM were eligible for participation. Moreover, the eligible participants should have a sedentary lifestyle and an impaired glucose homeostasis based on one or a combination of criteria including impaired fasting glucose, impaired glucose tolerance, HbA1c ≥ 5.7 and ≤ 6.4% (≥39 and ≤46 mmol/mol) and reduced glucose clearance rate ≤ 360 ml/min/m^2 indicating insulin resistance calculated by the Oral Glucose Insulin Sensitivity (OGIS) model (27). Detailed eligibility criteria are presented in electronic supplementary material (ESM) **table 1**.

Procedures

After informed consent, participants were randomly assigned to two 14-day intervention periods in which each participant received either dapagliflozin (10 mg/day) or placebo, separated by a 6 – 8 weeks washout. Main measurements were performed on day 12 – 14 of each intervention period during which participants stayed at the research facility. Study medication was taken once daily in the morning after awakening, and was continued during the end-of-treatment measurements. A detailed overview of the schedule of measurements can be seen in **ESM figure 1**.

Two days prior to their stay, participants were instructed to refrain from strenuous physical activities and alcohol consumption. On day 12, participants arrived at the research facility at 5 pm and entered a respiration chamber at 6.30 pm where they received a standardized dinner at 7 pm. Detailed information about the provided

meals can be found in the ESM. The respiration chamber is a small room equipped with whole-chamber indirect calorimetry (Omnical, Maastricht Instruments, Maastricht, the Netherlands (28)) to continuously measure oxygen consumption and carbon dioxide production. At 9 pm, participants completed the Macronutrient and Taste Preference Ranking Task (MTPRT (29)) to determine their food preferences and at 10.30 pm the lights of the respiration chamber were switched off and participants were instructed to sleep.

On day 13, participants were woken up at 6.45 am. During the day, participants stayed in the respiration chamber and followed a strict protocol consisting of subsequent blood draws, meals, and low-intensity activity exercises. Participants followed activity exercises to mimic physical activity levels in free-living conditions. Blood samples were collected at 17 time points spread over 24 hours, see ESM for an exact overview of the blood samples. Standardized meals were provided at 8 am, 12 pm, 4 pm, and 6 pm directly after the corresponding blood draw. Lights were switched off at 10.30 pm and participants were instructed to sleep. Urine was collected for 24 hours in 2 different aliquots of 16 or 8 hours each to determine differences in daytime and night-time. Urinary nitrogen was measured to calculate protein oxidation and urinary glucose was measured to correct for glucose loss.

On day 14, participants were woken up at 6.30 am. After leaving the respiration chamber, magnetic resonance spectroscopy (MRS) was performed at 7.30 am to measure hepatic glycogen, lipid content, and lipid composition. Subsequently at 9 am, blood pressure was measured and a skeletal muscle biopsy was taken according to the Bergström method (30). Breakfast was provided at 10 am and lunch at 12 pm. At 4 pm, a second skeletal muscle biopsy was performed which was followed by a second hepatic glycogen MRS examination at 5 pm. After the final MRS measurement was completed, the study protocol ended. A more detailed description of the measurement methods can be found in the ESM.

Biochemical analyses

Biochemical analyses are described in the ESM.

Statistics

Results are presented as mean ± SEM unless stated otherwise. Shapiro–Wilk normality test was performed to evaluate normal distribution. To compare differences between dapagliflozin and placebo treatment, a 2-tailed paired Student's t-test was used for normally distributed data and a Wilcoxon paired signed-rank for not normally distributed data. Pearson's correlation coefficient analyses were performed to identify associations between outcome measures. Analyses were performed for n=14 unless specified otherwise. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 27.0.

RESULTS

Fourteen men and women completed the study (**ESM Figure S2)**. Baseline characteristics are presented in **table 1.** Out of the 14 participants, 13 participants were randomized based on OGIS ≤360 ml/min/m^2 (27), and one participant had a value of 363 ml/min/m^2. This individual was randomized based on the HbA1c criteria (≥ 5.7 % (39 mmol/mol) and HOMA-IR was 2.7, indicating insulin resistance (31). Among the 14 participants, 8 had prediabetes based on their fasting plasma glucose values (6.1 – 6.9 mmol/L) or their 2-hour plasma glucose values (7.8 - 11.1 mmol/L), and 6 had HbA1c levels ≥ 5.7 % (39 mmol/mol).

After 14 days of treatment, body weight was similar between dapagliflozin and placebo periods (dapagliflozin: 88.5±2.8, placebo: 88.1±2.7 kg, p=0.397, **ESM Figure 3A**). No differences were found in HbA1c (dapagliflozin: 5.5±0.1 (37.1±1.5), placebo: 5.6±0.1 (37.3±1.2) % (mmol/mol), p=0.780, **ESM Figure 3B**). Systolic blood pressure (dapagliflozin: 141.6±3.4, placebo: 147.9±3.2 mmHg, p=0.028, **ESM Figure 3C**) and diastolic blood pressure (dapagliflozin: 87.3±2.3, placebo: 91.1±2.4 mmHg, p=0.023, **ESM Figure 3D**) were lower after dapagliflozin treatment compared to placebo, whereas heart rate was similar (dapagliflozin: 64.7±4.0, placebo: 64.6±4.3 beats/min, p=0.556, **ESM Figure 3E**).

Table 1: Participant characteristics

Characteristic	**Mean ± SD or number Total (n=14)**
Sex, female/male	6/8
Age, years	66.3 ± 6.2
Weight, kg	89.1 ± 10.3
BMI, kg/m^2	30.0 ± 2.1
Fat mass, %	35.6 ± 7.4
eGFR, mL/min	99 ± 19
Fasting plasma glucose, mmol/L	5.8 ± 0.6
2-hour plasma glucose, mmol/L	7.7 ± 1.9
Fasting plasma insulin, pmol/L	100 ± 49
HbA1c, % / mmol/mol	5.5 ± 0.5/ 37.1 ± 5.3
Glucose clearance, ml/min/m^2	310 ± 36[a]
HOMA-IR	4.3 ± 0.6
Impaired glucose homeostasis, (n)	
Impaired fasting glucose, 6.1 – 6.9 mmol/L	3
Impaired glucose tolerance, 7.8 - 11.1 mmol/L	5
Elevated HbA1c, 5.7 – 6.4 %	6
Impaired insulin sensitivity, OGIS ≤ 360 ml/min/m^2	13

Participant characteristics at baseline. Values are given as means ± SD or number (n = 14). [a] n=13.

24-hour energy metabolism

Twenty-four-hour urinary glucose excretion rate was higher after dapagliflozin compared to placebo (dapagliflozin: 1.52±0.159, placebo: 0.004±0.0002 gram/hour, $p < 0.0001$, **figure 1A**), resulting in a total dapagliflozin-induced glucose excretion of ~36 gram in 24-hours. Glucose excretion rate after dapagliflozin was twice as high during daytime compared to night-time (daytime: 1.865±0.190, night-time: 0.889±0.127 gram/hour, **figure 1B and C**). No changes were observed in urine volume produced (dapagliflozin: 2060±177, placebo: 2143±145 ml/24-hour, p=0.571, **figure 1D**).

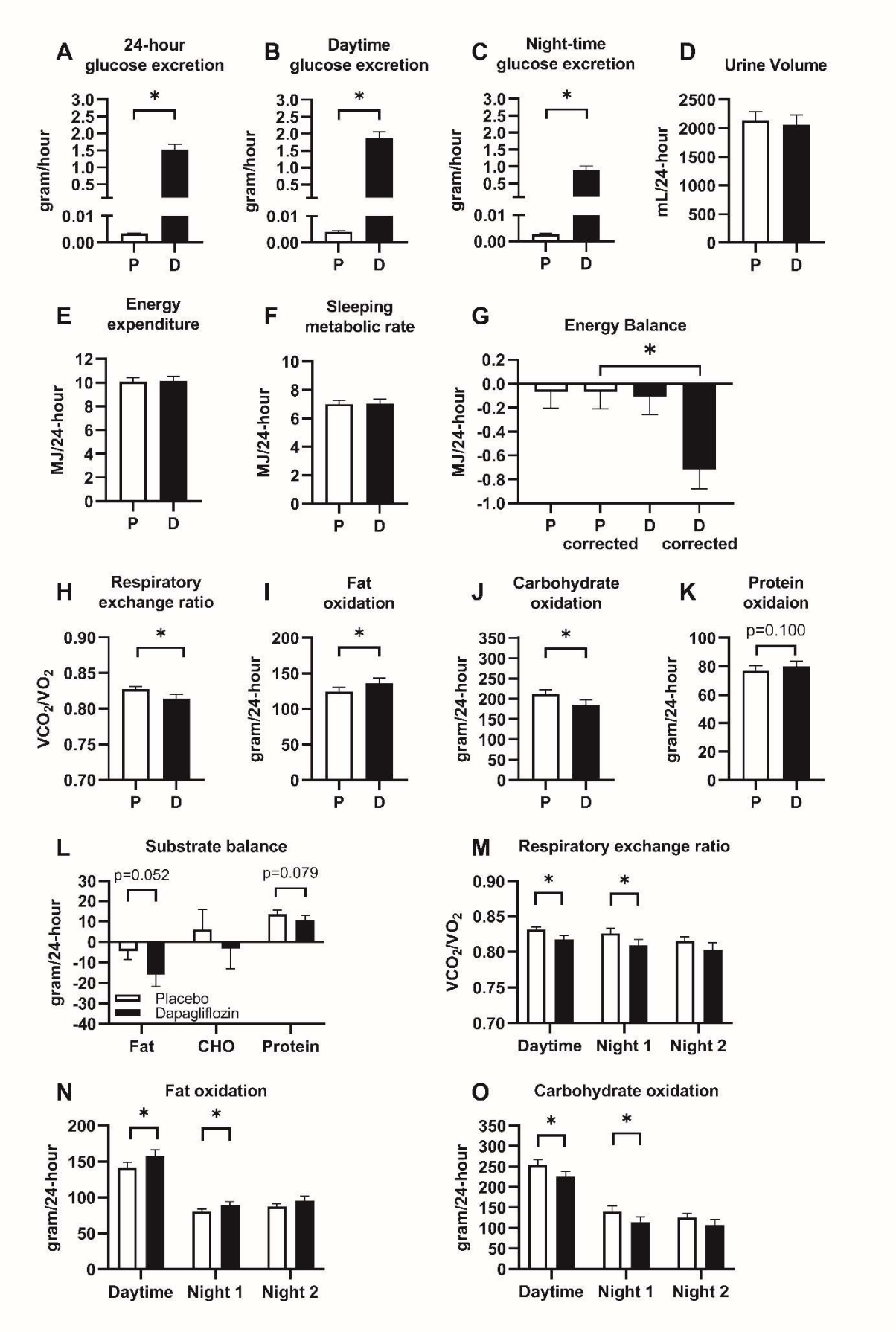
A
24-hour glucose excretion
gram/hour
P
D
B
Daytime glucose excretion
C
Night-time glucose excretion
D
Urine Volume
mL/24-hour
E
Energy expenditure
MJ/24-hour
F
Sleeping metabolic rate
G
Energy Balance
P corrected
D corrected
H
Respiratory exchange ratio
VCO2/VO2
I
Fat oxidation
gram/24-hour
J
Carbohydrate oxidation
K
Protein oxidaion
p=0.100
L
Substrate balance
p=0.052
p=0.079
Placebo
Dapagliflozin
Fat
CHO
Protein
M
Respiratory exchange ratio
Daytime
Night 1
Night 2
N
Fat oxidation
O
Carbohydrate oxidation

Figure 1: Effect of dapagliflozin on 24-hour energy metabolism. A: 24-hour glucose excretion; **B**: Daytime glucose excretion; **C**: Night-time glucose excretion; **D**: Urine volume; **E**: 24-hour energy expenditure; **F**: Sleeping metabolic rate; **G**: 24-hour energy balance; **H**: 24-hour respiratory exchange ratio; **I**: 24-hour fat oxidation; **J**: 24-hour carbohydrate oxidation; **K**: 24-hour protein oxidation; **L**: 24-hour substrate balance, for carbohydrate (CHO) balance only the values corrected for glucose excretion are presented; **M**: Day- and night-time respiratory exchange ratio; **N**: Day- and night-time fat oxidation; **O**: Day- and night-time carbohydrate oxidation. White bars represent placebo (P), and black bars represent dapagliflozin (D). * Indicates statistical significance (p<0.05). Data are expressed as mean±SEM. CHO; carbohydrate.

Twenty-four-hour energy expenditure was similar between dapagliflozin and placebo (dapagliflozin: 10.2±0.3, placebo: 10.1±0.4 MJ/24-hour, *p*=0.444, **figure 1E**). Similarly, sleeping metabolic rate did not differ between the treatment periods (dapagliflozin: 7.1±0.3, placebo: 7.0±0.3 MJ/24-hour, *p*=0.728, n=11, **figure 1F**). During the stay in the respiration chamber, energy intake was kept similar during the two study periods, and energy balance was not different between the treatment conditions (dapagliflozin: -0.11±0.15, placebo: -0.07±0.14 MJ/24-hour, p=0.654, **figure 1G**). However, after correction for the glucose loss, energy balance was significantly different between dapagliflozin and placebo treatment (dapagliflozin: -0.71±0.16, placebo: -0.07±0.14 MJ/24-hour, p<0.0001, **figure 1G**).

Twenty-four-hour respiratory exchange ratio (RER), reflecting the relative contribution of fat and carbohydrate oxidation, was numerically lower after dapagliflozin treatment (dapagliflozin: 0.814±0.006, placebo: 0.827±0.004, *p*=0.051, **figure 1H**). Similarly, 24-hour fat oxidation was significantly higher (dapagliflozin: 136.1±7.6, placebo: 124.3±6.0 gram/24-hour, *p*=0.043, **figure 1I**) and carbohydrate oxidation was significantly lower after dapagliflozin treatment (dapagliflozin: 185.6±12.0, placebo: 211.7±10.3 gram/24-hour, *p*=0.048, **figure 1J**). Protein oxidation was numerically higher after dapagliflozin treatment but did not reach statistical significance

(dapagliflozin: 80.0±3.7, placebo 76.8±3.7 gram/24-hour, p=0.100, **figure 1K**). As a result, fat balance tended to be more negative (dapagliflozin: -16.0±5.9, placebo: -4.6±4.1 gram/24-hour, *p*=0.052, **figure 1L**) and carbohydrate balance was more positive after dapagliflozin treatment (dapagliflozin: 33.0±10.4, placebo 6.1±9.8 gram/24-hour, *p*=0.048). However, after correction for glucosuria, the carbohydrate balance was not significantly different between dapagliflozin and placebo (dapagliflozin: -3.4±9.8, placebo: 6.0±9.8 gram/24-hour, *p*=0.551, **figure 1L**), illustrating the tight regulation of 24-hour carbohydrate balance. Protein balance was numerically lower after dapagliflozin compared to placebo, but this difference was not statistically significant (dapagliflozin: 10.5±2.4, placebo: 13.5±2.0 gram/24-hour, p=0.079, **figure 1L**).

Energy metabolism measured during daytime and night-time

Daytime RER (from 7 am to 10.30 pm) was significantly lower after dapagliflozin treatment compared to placebo (dapagliflozin: 0.818±0.005, placebo: 0.831±0.004, *p*=0.046, **figure 1M**). RER measured during the first night in the respiration chamber (night 1, from 12 am to 5.30 am) was significantly lower after dapagliflozin treatment (dapagliflozin: 0.809±0.008, placebo: 0.826±0.007, respectively, *p*=0.019), whereas RER during the second night in the respiration chamber (night 2, from 12 am to 5.30 am) was not statistically significantly different between dapagliflozin and placebo (dapagliflozin: 0.803±0.009, placebo: 0.816±0.006, *p*=0.125, n=11), probably due to the higher fat content (45 energy%) of the meals provided in the respiration chamber during the day before night 2. Fat oxidation during daytime and night 1 were significantly higher, whereas during night 2 fat oxidation was numerically higher, but did not reach statistical significance, after dapagliflozin treatment versus placebo (daytime: 156.8±9.1 versus 141.7±7.4 gram/24-hour, *p*=0.031, n=14; night 1: 89.5±4.8 versus 79.7±4.2 gram/24-hour, *p*=0.039, n=14; night 2: 95.3±6.2 versus 87.3±4.1 gram/24-hour in dapagliflozin versus placebo, *p*=0.110, n=11, **figure 1N**). Carbohydrate oxidation during daytime and night

1 were significantly lower, whereas the lower night 2 carbohydrate oxidation did not reach statistical significance, after dapagliflozin treatment versus placebo (daytime: 224.5±13.1 versus 254.4±11.9 gram/24-hour, *p*=0.050, n=14; night 1: 114.8±12.6 versus 140.3±13.9 gram/24-hour, p=0.018, n=14; night 2: 106.9±14.0 versus 124.7±10.8 gram/24-hour in dapagliflozin versus placebo, *p*=0.120, n=11, **figure 1O**).

Hepatic and skeletal muscle glycogen content

Overnight fasting total hepatic glycogen, corrected for liver volume, was numerically lower after dapagliflozin treatment, but did not reach statistical significance between treatment periods (dapagliflozin: 0.169±0.015, placebo: 0.188±0.016 arbitrary units (AU), *p*=0.129, n=12, **figure 2C**, see **figure 2A** also showing the results when liver volume is not taken into account). Liver volume measured in the morning (p=0.638, n=12, **figure 2B**), or the afternoon was not affected by dapagliflozin (p=0.282, n=7, **figure 2E**). When comparing the change in total hepatic glycogen (corrected for liver volume), measured in the morning (7.30 am) versus afternoon (5 pm), there was no difference between the dapagliflozin and placebo periods (dapagliflozin: -12.2±5.0, placebo: -11.7±7.3 %, *p*=0.966, n=7, **figure 2F,** see **figure 2D** also showing the results when liver volume is not taken into account).

Morning and afternoon skeletal muscle glycogen levels did not differ between dapagliflozin and placebo (morning: 371.7±22.8 versus 332.9±27.9 nmol/mg, *p*=0.250, n=10; afternoon: 340.5±24.3 versus 368.8±13.1 nmol/mg in dapagliflozin versus placebo, *p*=0.191, n=10; **figure 2G**). However, after dapagliflozin treatment, skeletal muscle glycogen levels numerically declined from morning to afternoon (morning: 371.7±22.8, afternoon: 340.5±24.3 nmol/mg, *p*=0.058, n=10, **figure 2G**), whereas after placebo skeletal muscle glycogen levels numerically increased from morning to afternoon (morning: 332.9±27.9, afternoon: 368.8±13.1 nmol/mg, *p*=0.116, n=10, **figure 2G**). Delta glycogen levels (afternoon minus morning) were negative upon dapagliflozin (-31.1±14.3 nmol/mg) and positive upon placebo (35.9±20.6 nmol/mg),

and there was a trend towards a significant difference between the changes in glycogen during the day comparing dapagliflozin and placebo periods (p=0.055, n=10, **figure 2H**).

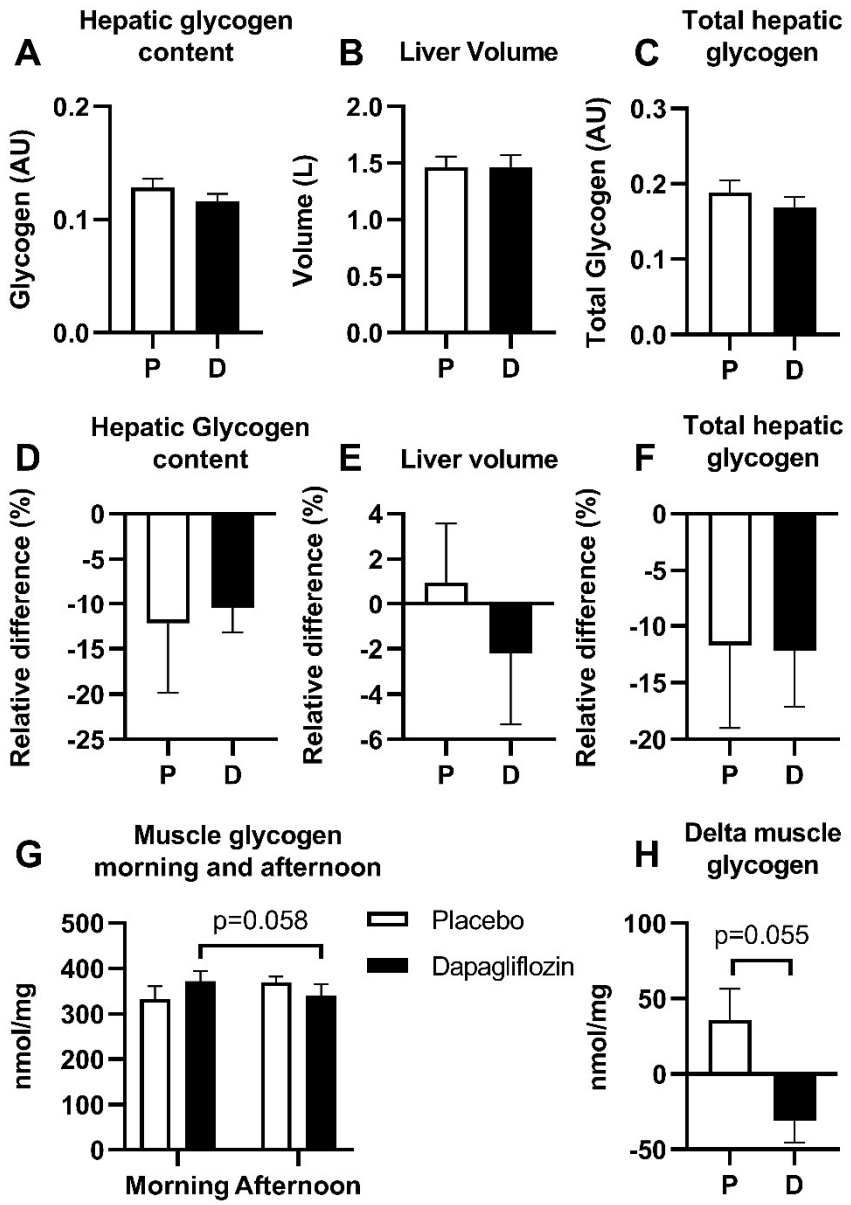

Figure 2: Effect of dapagliflozin on hepatic and skeletal muscle glycogen content. A: Hepatic glycogen content, not corrected for liver volume, measured in the morning; **B**: Liver volume measured in the morning; **C**: Total hepatic glycogen, corrected for liver volume, measured in the morning; **D**: Relative difference between morning and afternoon in hepatic glycogen content, not corrected for liver volume; **E**: Relative difference between morning and afternoon in liver volume; **F**: Relative difference between morning and afternoon in total hepatic glycogen, corrected for liver volume; **G**: Skeletal muscle glycogen measured in the morning and afternoon; **H**: Delta (afternoon minus morning) skeletal muscle glycogen. For A–C, n=11; for D–F, n=7; for G–H, n=10. White bars represent placebo (P), and black bars represent dapagliflozin (D). * Indicates statistical significance ($p<0.05$). Data are expressed as mean ± SEM.

24-hour blood values

During the stay in the respiration chamber, blood samples were taken every 2 hours during daytime (08 am–10 pm) and hourly during night-time (11 pm–07 am). Fasting plasma glucose at 8 am after an overnight fast was not different between treatment periods (dapagliflozin: 5.6±0.1, placebo: 5.7±0.2 mmol/L, p=0.346, **figure 3A**). However, the area under the curve (AUC) for 24-hour plasma glucose profiles was lower after dapagliflozin treatment (p=0.017) and tended to be lower during daytime and night-time (*p*=0.064 and p=0.056, respectively). Overnight fasting free fatty acid levels were not different between dapagliflozin and placebo (dapagliflozin: 331.1±31.0, placebo: 307.3±23.7 µmol/L, p=0.463, **figure 3B**). However, AUC for 24-hour free fatty acid profiles, as well as day- and night-time profiles, were significantly higher after dapagliflozin (p=0.002, *p*=0.014, and p=0.005 for 24-hour, daytime, and night-time, respectively). Overnight fasting plasma insulin levels measured at 8 am were similar between dapagliflozin and placebo (dapagliflozin: 10.1±1.0, placebo: 10.3±1.2 mU/L, p=0.749, **figure 3C**), whereas AUC for plasma insulin during daytime was numerically lower on dapagliflozin versus placebo (p=0.074). Fasting free glycerol levels measured at 8 am after an overnight fast were not different between treatments (dapagliflozin: 25.2±3.3, placebo: 30.5±2.8 µmol/L, p=0.109, n=12, **figure 3D**), and similarly, AUC for 24-hour free glycerol profiles, as well as day- and night-time profiles, were not different between dapagliflozin and placebo (p=0.781, *p*=0.946, and p=0.847 for 24-hour, daytime, and night-time, respectively, n=12). Plasma ß-hydroxybutyrate levels during night-time are shown since β-hydroxybutyrate levels were below the detection limit during the daytime; AUC for plasma β-hydroxybutyrate was significantly higher after dapagliflozin treatment as compared to placebo (p=0.012, **figure 3E**). Also, AUC for serum urea levels night-time was significantly higher after dapagliflozin treatment as compared to placebo (p=0.016, **figure 3F**).

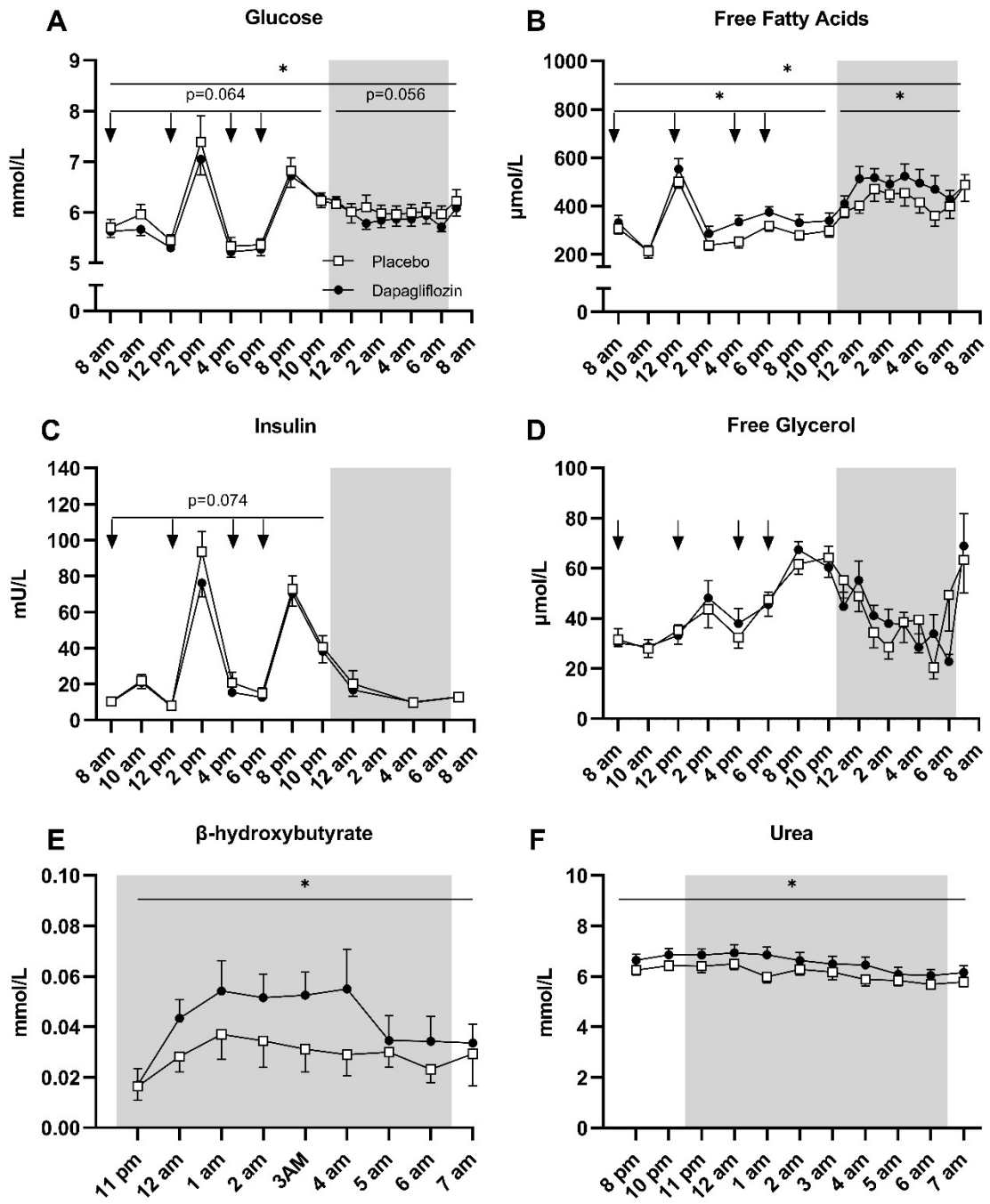

Figure 3: Effect of dapagliflozin 24-hour blood profiles. A: Glucose; **B**: Free fatty acids; **C**: Insulin; **D**: Free glycerol; **E**: β-hydroxybutyrate; **F**: Urea. White squares represent placebo, black dots represent dapagliflozin. Grey area represents the sleeping period and the arrows indicate meals. * Indicates statistical significance ($p<0.05$). Data are expressed as mean ± SEM.

Skeletal muscle mitochondrial oxidative capacity

Mitochondrial oxidative capacity was assessed at 9.15 am in permeabilized skeletal muscle fibres. ADP-stimulated (state 3) respiration on a lipid-derived substrate (malate + octanoyl-carnitine (MO): 30.5±2.1 versus 29.0±2.0 pmol/mg/s in dapagliflozin versus placebo, p=0.237, **figure 4A**) and upon addition of glutamate (MOG: 44.3±2.3 versus 42.6±2.3 pmol/mg/s in dapagliflozin versus placebo, p=0.209, **figure 4C**) were not different between treatment periods. After the addition of succinate, state 3 respiration was numerically higher after dapagliflozin compared to placebo (MOGS: 68.2±3.2 versus 64.4±3.2 pmol/mg/s in dapagliflozin versus placebo, p=0.071, **figure 4C**). No differences were observed in state 3 respiration without octanoyl-carnitine (MG: 40.4±1.9 versus 41.3±2.5 pmol/mg/s, p=0.598; MGS: 72.7±2.7 versus 73.0±3.7 pmol/mg/s in dapagliflozin versus placebo, p=0.881, **figure 4B and C**). Maximal FCCP-induced uncoupled respiration, which reflects the maximal oxidative capacity of the electron transport chain, was significantly higher after dapagliflozin treatment compared to placebo (dapagliflozin: 87.6±5.4, placebo: 78.1±5.5 pmol/mg/s, p=0.007, **figure 4D**). State 4o respiration, reflecting a proton leak, was not different between treatment periods (dapagliflozin: 22.0±1.3, placebo: 21.2±1.4 pmol/mg/s, p=0.560, **figure 4E**).

The protein content of OXPHOS complex III was significantly higher after dapagliflozin compared to placebo (dapagliflozin: 1.19±0.06, placebo: 0.98±0.06 AU, *p*=0.047, **figure 4F**), whereas OXPHOS complex V was numerically higher after dapagliflozin, but did not reach statistical significance (dapagliflozin: 1.20±0.05, placebo: 1.03±0.06 AU, *p*=0.067). None of the other complexes were statistically different between the treatment arms (Complex I: 1.33±0.12 versus 1.08±0.12 AU, *p*=0.137; Complex II: 1.29±0.10 versus 1.11±0.10 AU, *p*=0.133; Complex IV: 1.15±0.07 versus 1.06±0.09 AU, *p*=0.363 in dapagliflozin versus placebo).

Since mitochondrial respiration has been shown to be controlled by PGC1α (32), levels of PGC1α protein were measured in the muscle biopsies. PGC-1α protein content did

not differ between dapagliflozin and placebo (1.14±0.18 versus 1.00±0.15 AU, *p*=0.239, n=12, **figure 4G**). No significant correlation was observed between change in PGC-1α levels and change in maximal FCCP-induced uncoupled respiration comparing the dapagliflozin and placebo periods (r=0.476, p=0.118, n=12, **figure 4H**).

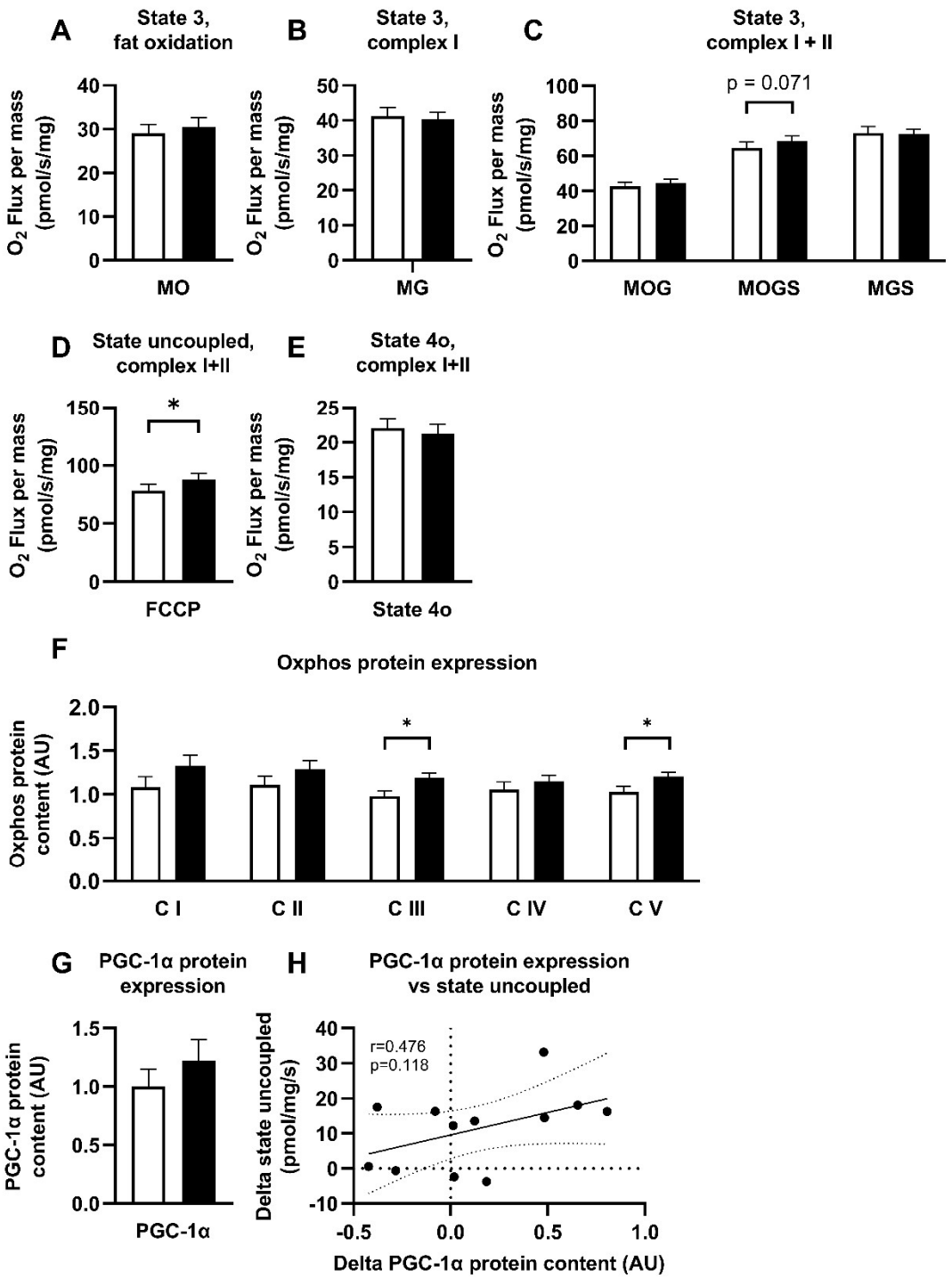

Figure 4: Effect of dapagliflozin on *ex vivo* skeletal muscle mitochondrial respiration measured in the morning. A: ADP-stimulated state 3 respiration upon a lipid-derived substrate (MO); **B**: ADP-stimulated state 3 respiration upon Complex I substates (MG); **C**: ADP-stimulated state 3 respiration upon parallel electron input to both Complex I and Complex II (MOG, MOGS, and MGS); **D**: Maximal uncoupled respiration upon FCCP; **E**: Oligomycin induced respiration; **F**: Mitochondrial protein expression of oxidative phosphorylation (OXPHOS) complex I, complex II, complex III, complex IV, and complex V; **G**: Protein expression

of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α); **H**: Correlation of change in PGC-1α protein expression and change in maximal uncoupled respiration upon FCCP. White bars represent placebo, black bars represent dapagliflozin. * Indicates statistical significance (p<0.05). Data are expressed as mean±SEM. C, complex; FCCP, carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone; M, malate; O, octanoyl-carnitine; G, glutamate; S, succinate; Oxphos, oxidative phosphorylation.

Hepatic lipid content and composition

Five out of 14 participants had a hepatic lipid content ≥ 5.0%, which is generally used as the cut-off to define non-alcoholic fatty liver disease (NAFLD). Dapagliflozin did not affect hepatic lipid content (dapagliflozin: 6.0±2.4, placebo: 6.4±2.6 %, *p*=0.510, **ESM Figure 4A**). Similarly, the fraction of SFA, MUFA, and PUFA were not different after dapagliflozin treatment (SFA: 41.0±1.4 versus 40.7±2.6 %, *p*=0.890, n=10; MUFA: 43.6±1.9 versus 42.1±2.5 %, *p*=0.483, n=7; PUFA: 16.4±1.8 versus 18.0±2.6 %, *p*=0.517, n=7 in dapagliflozin versus placebo, **ESM Figure 4B, C and D**).

Intramyocellular lipid content

Dapagliflozin did not affect IMCL content in all fibers, type 1 fibers, or type 2 fibers (All: 0.51±0.22 versus 0.46±0.13 %, *p*=0.972; Type 1: 0.61±0.25 versus 0.56±0.17 %, *p*=0.650; Type 2: 0.43±0.20 versus 0.39±0.11 %, *p*=0.701 in dapagliflozin versus placebo, n=13, **ESM Figure 5**).

Food preferences

To assess whether there were differences in food preferences, the participants filled out MTPRT on the evening of day 12. The relative preference for sweet products did not differ between treatment periods (dapagliflozin: 2.64±0.8, placebo: 2.65±0.9, *p*=0.809, **ESM Figure 6A**). Similarly, the relative preference for high-carbohydrate, high-fat, high-protein, or low-energy products was similar (p > 0.05, **ESM Figure 6B**). No differences were found in liking ratings (VAS-scale 0 – 100) for sweet, savory, high-carbohydrate, high-fat, high-protein, or low-energy products (p > 0.05, **ESM Figure 6C**).

Safety

No serious adverse events or events of diabetic ketoacidosis were reported.

DISCUSSION

We here show that dapagliflozin can exert similar adaptive metabolic changes in energy metabolism in prediabetic insulin resistant individuals as in T2DM patients after only 14 days of treatment and no detectable effect on body weight. To compensate for the loss of glucose, it has been shown that SGLT2i treatment increase EGP in the overnight fasted state in patients with T2DM (10-12, 18, 19). Until now only one study investigated the direct contribution of gluconeogenesis and glycogenolysis on SGLT2i-induced EGP (33). In that study, a single dose of dapagliflozin increased EGP in healthy control individuals, mainly by increasing gluconeogenesis, while the rate of glycogenolysis was not affected. However, the plasma levels of the gluconeogenic substrates were not affected by dapagliflozin. Here, we investigated if the glucosuria induced by dapagliflozin was compensated by a reduction in overnight hepatic glycogen levels. We found that after 14 days of treatment, overnight hepatic glycogen was numerically lower after dapagliflozin treatment as compared to placebo but the difference did not reach statistical significance. The lack of a significant effect on hepatic glycogen content after dapagliflozin treatment may indicate that gluconeogenesis is predominantly responsible for increased EGP. In line with this suggestion, we observed an increase in urea which is reflective of enhanced gluconeogenesis. On the other hand, we did not observe changes in levels of free glycerol, which is a precursor for gluconeogenesis. Alternatively, considering that the glucose loss overnight only summed up to a total of ~7 – 8 gram, it may be difficult to detect a corresponding change in hepatic glycogen levels, and the lack of effect on hepatic glycogen content may also indicate that the power of our study was insufficient to detect changes in hepatic glycogen content. Furthermore, it is possible that an increase in gluconeogenesis can contribute to both retaining glycogen content

(or blunting the decline), as well as maintaining plasma glucose levels. Further investigations are needed to elucidate the exact contribution of gluconeogenesis and glycogenolysis to EGP after SGLT2i treatment.

Also skeletal muscle glycogen content was not different between dapagliflozin and placebo in the overnight fasted state. Interestingly, skeletal muscle glycogen content increased from morning to afternoon by ~18% after placebo, which is in line with previously observed diurnal changes in skeletal muscle glycogen levels due to postprandial glycogen storage (34). However, there was an opposite trend from morning to afternoon, an ~8% decrease in glycogen content, after dapagliflozin treatment. These findings may suggest that upon excess urinary glucose loss, dietary carbohydrates may be preferentially directed toward the liver, as hepatic glycogen levels may be more crucial for glucose homeostasis. This suggestion is in line with the similar change in hepatic glycogen levels from the morning to the afternoon comparing placebo and dapagliflozin periods. However, it must be noted that we could only measure hepatic glycogen both in the morning and afternoon in a subset of the volunteers. Furthermore, it could be speculated that skeletal muscle glycogen levels are restored by the enhanced EGP during the night, which is in line with our previous observation that overnight fasted non-oxidative glucose disposal (mainly glycogen storage) was increased in T2DM patients treated with dapagliflozin (10). Larger studies with glycogen measurements around the clock are needed to investigate the effect of SGLT2i on substrate fluxes in muscle and liver.

We hypothesized that SGLT2i treatment would lead to an improvement in nocturnal fat oxidation in prediabetic insulin resistant individuals. Indeed, nocturnal fat oxidation was increased while carbohydrate oxidation was decreased during the first night in the respiration chamber. During the second night in the respiration chamber, the difference in fat oxidation between dapagliflozin treatment and placebo was blunted. Most likely, the higher fat oxidation in the placebo arm during the second night was

due to the diet provided during the stay in the respiration chamber, which was relatively low in carbohydrates (38 energy%) and high in fat (45 energy%) and may have further stimulated fat oxidation affecting both treatment periods. Interestingly, despite the relative high-fat diet, fat balance was more negative during dapagliflozin treatment.

In line with improved fat oxidation *in vivo*, especially in the nocturnal state, we also observed an improved *ex vivo* skeletal muscle mitochondrial oxidative capacity after dapagliflozin treatment. An improved mitochondrial oxidative capacity may be clinically relevant as a decreased mitochondrial oxidative capacity is associated with insulin resistance and T2DM (35). There are (pre)clinical results showing that both caloric restriction and SGLT2i can activate the AMPK-SIRT1-PGC-1α signalling pathway (36-39), which is involved in many processes, including mitochondrial biogenesis (32). However, in our study, PGC-1α levels in the morning were not significantly influenced by dapagliflozin treatment and we did not observe a significant correlation between changes in PGC-1α levels and changes in maximal mitochondrial oxidative capacity comparing the dapagliflozin and placebo periods. In conclusion, more studies are needed in order to conclude the importance of the AMPK-SIRT1-PGC-1α pathway for mitochondrial function after SGLT2i treatment.

The glucose loss reported in this study was ~36 gram/24-hour, resulting in an energy deficit of ~600 kJ/24-hour, which represents only 6% of the daily energy intake. Interestingly, this mild glucose loss did result in lower 24-hour plasma glucose levels. These results illustrate that mild glucose loss can exert strong metabolic adaptations in substrate metabolism in organs well distal to the direct pharmacological impact of the drug. It is well known that in the insulin resistant state, metabolic disturbances in energy and substrate metabolism and impaired mitochondrial function are present (40). Therefore, our results highlight the potential of SGLT2i to systemically restore such metabolic disturbances under insulin resistant conditions.

A limitation of our study is the short duration of dapagliflozin treatment and the low number of participants. Although we observed positive metabolic adaptations without changes in body weight, the outcomes might be different after several months of SGLT2i treatment. It has been shown that SGLT2i increase caloric intake after 6 months of treatment (41), possibly as a compensatory mechanism to overcome the urinary glucose losses. Here we also investigated whether dapagliflozin-induced glucose loss would lead to a change in food preferences in favour of carbohydrate intake, but we could not confirm previous findings (42). Regardless of changes in caloric intake, it should be noted that interventions that increase energy turnover, such as cold exposure (43) and exercise (44), generally are associated with improvements in metabolic health, also when no weight loss is achieved.

CONCLUSION

In conclusion, we show that 2 weeks of dapagliflozin treatment in prediabetic insulin resistant individuals lowered 24-hour glucose levels, and improved 24-hour and nocturnal fat oxidation, as well as *ex vivo* mitochondrial oxidative capacity without significant changes in hepatic glycogen stores. Future long-term clinical trials should investigate if the calorie restriction-like effects of SGLT2i on energy and substrate metabolism may underly the reported organ protective effects of SGLT2i that are also observed in people with chronic kidney disease and heart failure without T2DM. Furthermore, the exact role of overnight changes in hepatic glycogen and/or gluconeogenesis in regulating energy metabolism needs further investigation.

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Data availability

The datasets that were obtained in this study can be made available by the corresponding author upon reasonable request.

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Authors' relationships and activities

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Contribution statement

A.V., M.d.L., R.E., J.O., V.B.S.-H., E.P., and P.S. designed the experiments. A.V., C.A., Y.O.d.K., E.E.T., J.J., E.M.-K., and G.S. performed the measurements. A.V., J.M., J.J., V.B.S.-H., E.P., and P.S. were involved in the data analysis. A.V., J.O., and P.S drafted the manuscript. All authors reviewed and edited the manuscript. P.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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SUPPLEMENTARY MATERIAL FOR CHAPTER 6

PROCEDURES

Randomization

A randomization list was generated by an independent researcher prior to the start of the study using the website Randomizer.org. Treatment sequences (dapagliflozin:placebo or placebo:dapagliflozin) were generated using blocks of equal size to prevent treatment-order effects. Both researchers and participants were blinded for treatment sequence. When participants were found eligible to participate, randomization codes were assigned strictly sequentially by the coordinating investigator to assign each participant to a specific treatment sequence.

Blood Sampling

Blood samples were drawn at 17 timepoints spread 24 hours. Blood was drawn once every two hours from 8 am to 10 pm (8 am, 10 am, 12 pm, 2 pm, 4 pm, 6 pm, 8 pm, and 10 pm) and hourly between 11 pm and 7 am the next day (11 pm, 12 am, 1 am, 2 am, 3 am, 4 am, 5 am, 6 am, and 7 am). Participants were not awakened while blood samples were taken during the sleeping period.

Meals

Energy intake for dinner on day 12 was calculated by multiplying resting metabolic rate, determined using the Harris-Benedict formula (1) with an activity factor of 1.4. Dinner consisted of ~46 energy% (E%) carbohydrates, ~39 E% fat, and ~14 E% protein. For day 13, the energy intake was determined from the sleeping metabolic rate (SMR) of the first night, multiplied by an activity factor of 1.5. The participants received 3 meals and a snack. Breakfast accounted for ~20 E%, lunch for ~25 E%, the snack for

~10 E% and, dinner for ~45 E%. Daily macronutrient composition was ~38 E% carbohydrates, ~45 E% fat and, ~15 E% protein. For day 14, energy intake was kept the same for all participants, with ~52 E% carbohydrates, ~33 E% fat and, ~16 E% protein. All meals were completely consumed by the participants. No other food or drinks containing calories were consumed.

Muscle Biopsies

At 9.15 am and 4 pm of day 14, a muscle biopsy was taken from the vastus lateralis muscle under local anaesthesia (1% lidocaine, without epinephrine) according to the Bergström method (2). The muscle biopsy was divided into several parts. One part of the muscle was immediately placed in ice-cold preservation medium (BIOPS, OROBOROS Instruments, Innsbruck, Austria) and used for mitochondrial respiration analysis. The remaining parts were immediately frozen in melting isopentane and stored at -80 °C until further analysis.

Skeletal Muscle Mitochondrial Respiration

From the muscle biopsy, permeabilized muscle fibres were prepared as described elsewhere (3). Thereafter, oxygen consumption was measured using high-resolution respirometry (Oxygraph, OROBOROS Instruments) upon the addition of several substrates, as described previously (4, 5). To prevent oxygen limitation, the respiration chambers were hyper-oxygenated up to ~400 µmol/L O_2. Subsequently, two different multi-substrate/inhibition protocols were used in which substrates and inhibitors were added consecutively at saturating concentrations. State 2 respiration was measured after the addition of malate (4 mmol/L) plus octanoyl-carnitine (1 mmol/L) or malate (4 mmol/L) plus glutamate (10 mmol/L). Subsequently, an excess of 2 mmol/L of ADP was added to determine coupled (state 3) respiration. Coupled respiration was then maximized with convergent electron input through Complex I and

Complex II by adding succinate (10 mmol/L). Finally, the chemical uncoupler carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) was titrated to assess the maximal capacity of the electron transport chain (state 3u respiration) or oligomycin (2 µg/ml) was added to assess the respiration not coupled to ATP synthesis (state 4o respiration). The integrity of the outer mitochondrial membrane was assessed by the addition of cytochrome C (10 µmol/L) upon maximal coupled respiration. If cytochrome C increased oxygen consumption ≥ 15%, that measurement was excluded to assure the quality of the muscle mitochondrial measurement. All measurements were performed in quadruplicate.

Magnetic Resonance Imaging and Spectroscopy

Magnetic resonance spectroscopy (MRS) was used to quantify hepatic lipid content and fatty acid composition (fraction of hepatic saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), and poly-unsaturated fatty acids (PUFA)), and hepatic glycogen. Localized STEAM proton MRS (^{1}H-MRS) with VAPOR water suppression was used for *in-vivo* hepatic lipid measurements (Voxel: 30x30x30mm^{3}, TE: 20ms, TR: 4500ms, NSA 128, data points: 2048, bandwidth: 2000Hz). Water reference spectra for absolute quantification were acquired with the same settings as the lipid measurements, however with a NSA of 16 and without VAPOR. Carbon MRS (^{13}C-MRS) FID was used to determine hepatic glycogen. Spectra were acquired by using a 21x24cm 13C-quadrature detection surface coil (RAPID Biomedical GmbH, Germany). Spectra were acquired with the participant lying in the prone position (FID; TR: 280ms; NSA: 4096) using a block pulse, calibrated to achieve a 90-degree excitation angle at a distance of 8 cm from the coil All measurements were performed on a 3T MRI scanner (Achieva 3T-X, Philips Healthcare, Best, The Netherlands).

Hepatic Lipid Content and Composition

Hepatic lipid content and composition measurements took place on day 14 of each intervention period at 8.15 am after an overnight fast. Spectra were acquired and post-processed as described previously (6). Lipid content values are given as a T2 corrected ratio of the CH2 peak relative to the sum of the unsuppressed water peak and the CH2 peak, converted to weight/weight percentage (7, 8). Lipid composition is given as a fraction of SFA, MUFA, and PUFA, values were calculated as described previously (6).

Hepatic Glycogen Content

Hepatic glycogen determination took place on day 14 of each intervention period at 7.30 am after an overnight fast and at 5 pm in the postprandial state, 5 hours after lunch (see above). Post-processing was performed with an in-house developed MATLAB (MATLAB 2018b, The MathWorks, Inc., Natick, Massachusetts, United States) script. The two spectra from the same individual that were to be compared, were pre-processed simultaneously to ensure identical frequency alignment and phase correction. The area under the curve (AUC) of the glycogen signal was determined by integration of ± 5 PPM around the C1 doublet of glycogen at 100.5 PPM. A B1 map was determined in a phantom measurement with two-dimensional chemical shift imaging (multiple slices) and the sensitivity profile was taken into account by manually segmenting the liver on cross-sectional MRI images in a custom-written MATLAB script and multiplying the weighted amount of pixels in the liver with the respective sensitivity (based on the B1 sensitivity map). Thereafter, the AUC of the glycogen signal was divided by the weighted number of liver pixels. Coil loading was assumed to be identical between the measurements within the same individuals. Therefore, even without correction for coil loading the signal difference within an individual is directly proportional to the difference in glycogen concentration. Therefore, relative changes in hepatic glycogen were calculated and given as percentage change ((hepatic $glycogen_{7.30\ am}$ - hepatic $glycogen_{5\ pm}$)/ hepatic $glycogen_{5\ pm}$ * 100). Liver volume was

measured directly after the hepatic glycogen measurements by MRI. Analyses were performed manually on cross-sectional MRI images in MATLAB. Due to technical problems, hepatic glycogen and liver volume could not be assessed at all time points; therefore, hepatic glycogen analysis could be calculated for 12 participants at 7.30 am and for 7 participants at 5 pm.

Intramyocellular lipid content

For intramyocellular lipid (IMCL) content, the lipid area fraction was determined in 5 µm-thick muscle sections. Sections were mounted on glass slides and subsequently permeabilized for 5 min with 0.25% TX-100 in PBS (648466, Merck, Darmstadt, Germany), thereafter sections were incubated with primary antibodies against laminin (L9393; Sigma, St Louis, USA) and myosin heavy chain type I (A4.840; Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) for 60 min. Subsequently, sections were incubated for 90 minutes with the appropriate secondary antibodies conjugated with AlexaFluor350 and AlexaFluor555 and Bodipy 493/503 (D3922, Invitrogen-ThermoFisher, The Netherlands). Sections were mounted with Mowiol, covered with #1.5 coverslips, and stored in the dark until imaging with a Nikon E800 fluorescence microscope (Nikon, Amsterdam, The Netherlands) coupled to a Nikon DS-Fi1c colour CCD camera (Nikon) using NIS-Elements imaging software (Nikon). Type I fibers were identified based on positive myosin heavy chain type I staining, all other fibers were considered to be type II fibers. Images were analyzed for lipid area fraction using ImageJ (NIH, Bethesda, USA) (9) for all fibers and in a fiber type specific manner.

Skeletal muscle glycogen

Muscle tissue (~30 mg) was freeze-dried overnight and dissected at room temperature under a microscope to remove collagen and blood. HCl (1M) was added to the isolated muscle fibers (~5-7mg) followed by heating for 3 hours at 100°C.

Thereafter the samples were neutralized with Tris-KOH (119 mM Tris, 2.14M KOH) and centrifuged at 1000g for 10 min at 4°C. Finally, glucose concentration in mmol/L (HORIBA, Montpellier, France) was measured in the supernatant using a Pentra C400 (HORIBA, Montpellier, France) and values were corrected for dry tissue weight.

Body Composition

Total mass, fat mass, and lean mass were measured as part of the baseline characteristics by DXA using the Hologic Discovery scanner (Hologic).

Biochemical analysis

EDTA-plasma glucose (Horiba, Montpellier, France), free fatty acids (WAKO, Neuss, Germany), and ß-hydroxybutyrate (Randox, Crumlin, UK) levels were measured colorimetrically using a Cobas Pentra C400 analyzer (Horiba, Montpellier, France). Free glycerol was measured in EDTA-plasma on a spectrophotometer using the free glycerol reagent (Sigma, St Louis, USA). Insulin levels were measured in EDTA-plasma using an ELISA (Crystal Chem, Els Grove Village, USA). Urea (Roch, Mannheim, Germany) was measured in serum using the Roche/Hitachi Cobas c systems Urinary nitrogen levels were measured using the Vario Max (Elementar Analysensysteme GmbH, Langenselbold, Germany), and urinary glucose levels (Roche, Mannheim, Germany) using the Roche/Hitachi Cobas c systems. All samples from one participant were analysed within the same run.

Western Blots

PGC-1aα and OXPHOS protein expression was determined using Western blot analyses in Bioplex lysates (Bio-Rad Laboratories, Veenendaal, The Netherlands) of human muscle tissue as previously described (10). Equal amounts of protein were loaded on

Bolt 4–12%, Bis-Tris Plus, gels (Invitrogen by Thermo Fisher Scientific, Bleiswijk, The Netherlands) or on 4-15% Criterion TGX Stain-Free Protein Gels (BioRad Laboratories). Proteins were transferred to a nitrocellulose membrane with the Trans-Blot Turbo transfer system (Bio-Rad Laboratories). Blots were incubated with primary antibodies against PGC-1α (dilution 1:10.000, 516557, Calbiochem, Darmstadt, Germany) or a cocktail of mouse monoclonal antibodies directed against human OXPHOS proteins (dilution 1:10,000; ab110411, Abcam, Cambridge, UK). The target proteins were detected using secondary antibodies conjugated with IRDye800 and were quantified with the CLx Odyssey Near-Infrared Imager (Li-COR, Homburg, Germany).

Calculations

Sleeping metabolic rate was defined as three consecutive hours with the lowest energy expenditure during the sleeping period, calculated using Weir et al. formula (11). Twenty-four-hour energy expenditure, carbohydrate oxidation, and fat oxidation were calculated based on the measured average oxygen and carbon dioxide concentrations from the last 24-hour in the respiration chamber starting at 5.30 am using the Brouwer et al. methodology (12). Protein oxidation was calculated as 24-hour urinary nitrogen excretion * 6.25. Daytime energy metabolism was calculated from 7 am to 10.30 pm and night-time from 12 am to 5.30 am. Due to practical problems, energy metabolism during the second night in the respiration chamber could not be assessed for 3 participants; therefore, for those participants, 24 hour energy metabolism was based on 24 hours in the respiration chamber starting on the first night at 10 pm.

Sample size was determined based on a difference in nocturnal RER measured by whole-chamber indirect calorimetry. We expected a nocturnal RER upon dapagliflozin and placebo of 0.82 ± 0.04 and 0.85 ± 0.04, respectively, based on previous studies performed in our research facilities (4) and upon dapagliflozin treatment (13). With a

two-sided statistical significance level (α) of 0.05 and power (1-β) of 0.80 in a cross-over design with a correlation between groups of 0.6, the required number of participants would be n=14.

RESULTS

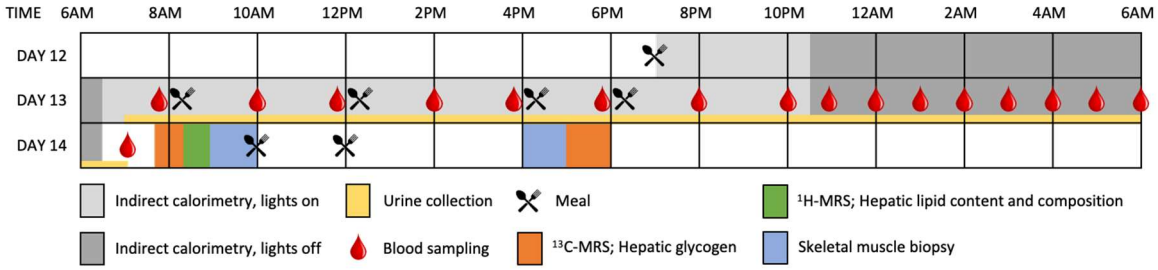

Figure 1: Study protocol with an overview of the in-house stay (day 12, 13, and 14). ^{1}H-MRS, proton magnetic resonance spectroscopy; ^{13}C-MRS, carbon magnetic resonance spectroscopy

CONSORT 2010 Flow Diagram

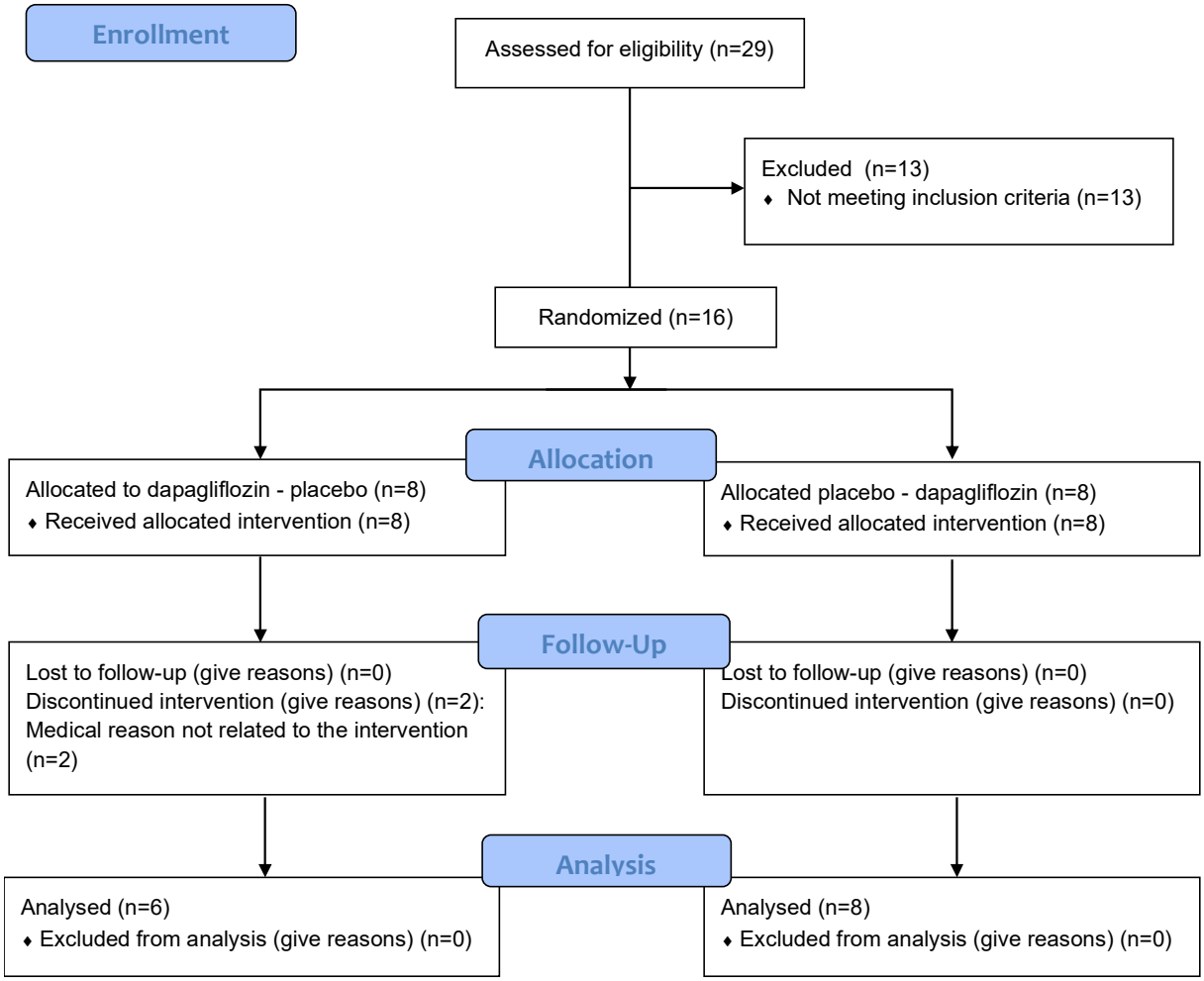

Figure 2: **Consort flow diagram of this trail.**

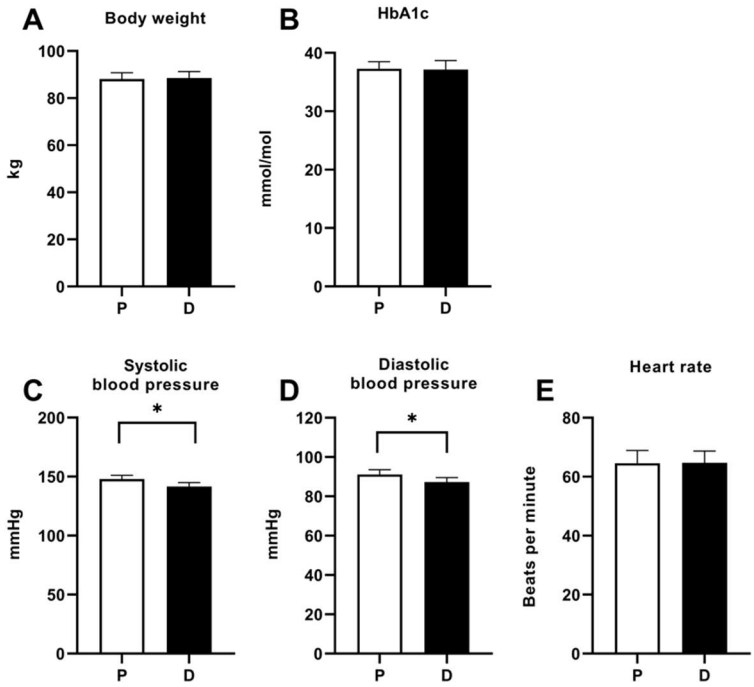

Figure 3: A: Body weight; **B**: HbA1c; **C**: Systolic blood pressure; **D**: Diastolic blood pressure; **E**: Heart rate. White bars represent placebo (P), and black bars represent dapagliflozin (D). * indicates statistical significance ($p<0.05$). Data are expressed as mean ± SEM.

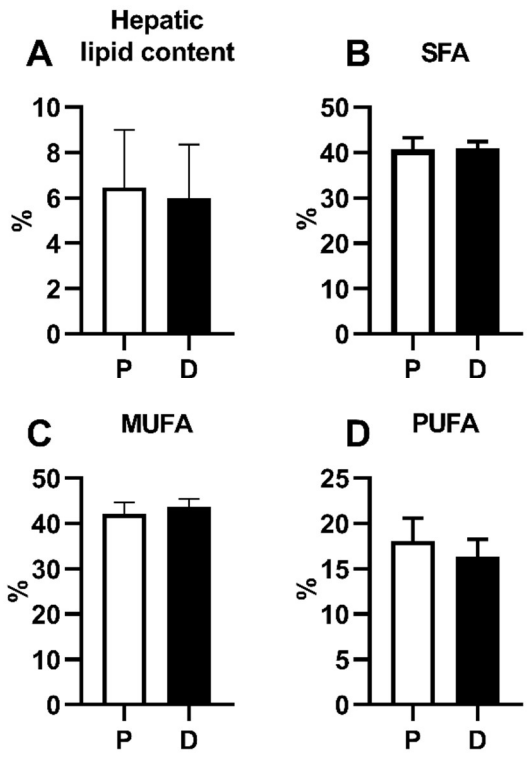

Figure 4: Effect of dapagliflozin on hepatic lipid content and composition. A: Hepatic lipid content; **B**: Saturated fatty acids; **C**: Monounsaturated fatty acids; **D**: Polyunsaturated fatty acids. For A, n = 14; for B, n = 12; for C and D, n = 8. White bars represent placebo (P), and black bars represent dapagliflozin (D). Data are expressed as mean ± SEM.

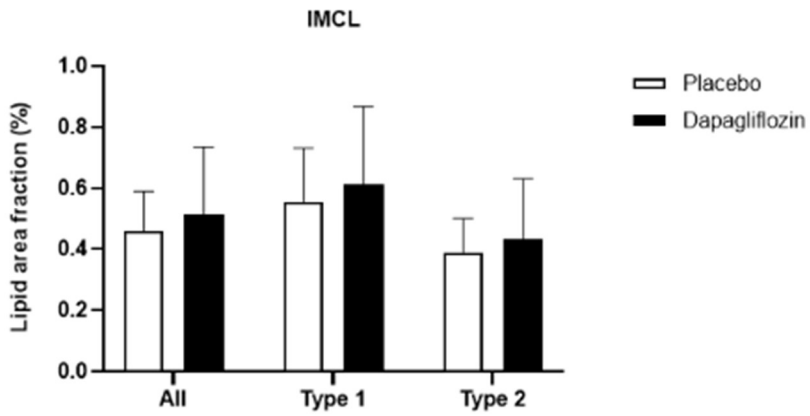

Figure 5: Effect of dapagliflozin on intramyocellular lipid (IMCL) content. n = 13. White bars represent placebo, and black bars represent dapagliflozin. Data are expressed as mean ± SEM.

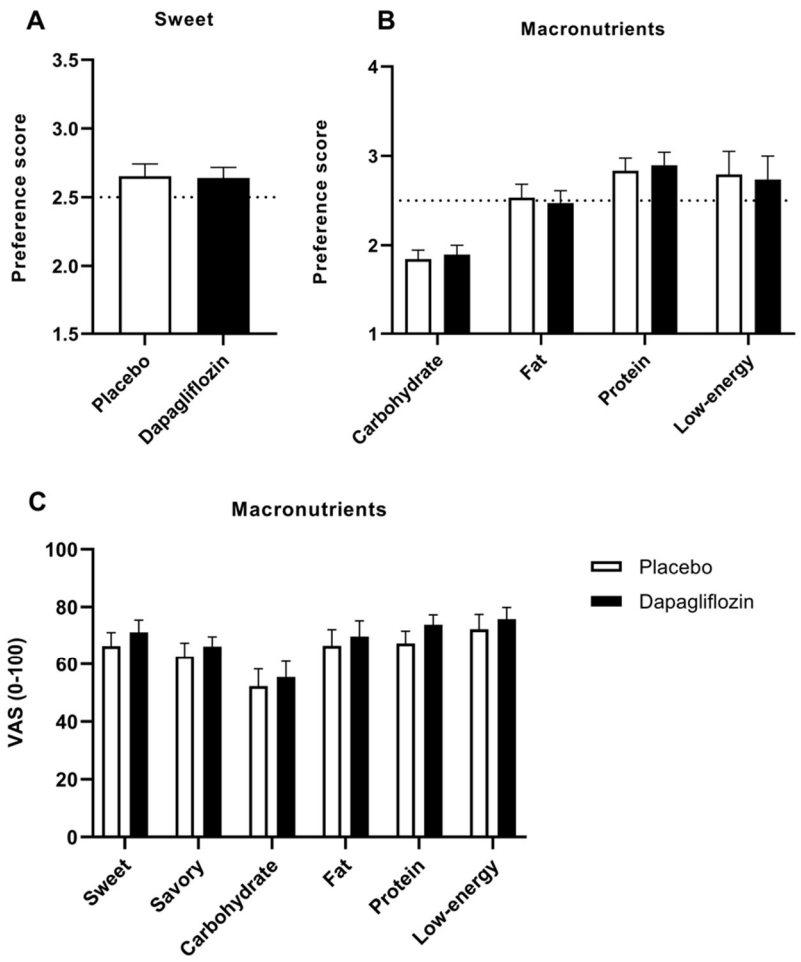

Figure 6: Effect of dapagliflozin on food preferences. A: Relative preference for sweet products; **B**: Relative preference for macronutrients; **C**: Liking for macronutrients. n = 12. White bars represent placebo (P), and black bars represent dapagliflozin (D). Data are expressed as mean ± SEM.

Table 1: Inclusion and exclusion criteria of the trail

Inclusion criteria	
1	Provision of signed and dated informed consent prior to any study specific procedures
2	Men aged ≥ 40 and ≤ 75 years and post-menopausal women (defined as at least 1 year post cessation of menses) aged ≥ 50 and ≤ 75 years
3	BMI ≥ 27 and ≤ 38 kg/m2
4	Sedentary lifestyle (not more than 2 hours of vigorous exercise per week)
5	Stable dietary habits
6	Impaired glucose homeostasis based on one or a combination of the following criteria: • Impaired Fasting Glucose (IFG): fasting plasma glucose ≥ 6.1 mmol/l and ≤ 6.9 mmol/l; • Impaired Glucose Tolerance (IGT): plasma glucose values ≥ 7.8 mmol/l and ≤ 11.1 mmol/l 120 minutes after consumption of the glucose drink during the 2h, 3-point OGTT; • HbA1c ≥ 39 and ≤ 46 mmol/mol / ≥ 5.7 and ≤ 6.4%; • Insulin Resistance: glucose clearance rate ≤ 360 ml/min/m2, as calculated by Oral Glucose Insulin Sensitivity 120 (OGIS120) model (14) based on the 2h, 3-point OGTT
Exclusion criteria	
1	Clinical diagnosis of Type 1 or 2 Diabetes Mellitus
2	Active cardiovascular disease: participants who experienced a heart attack in the last year, or participants who are currently under regular control of a physician for a heart condition
3	Weight gain or loss > 5 kg in the last 3 months, ongoing weight-loss diet (hypocaloric diet) or use of weight loss agents
4	Regular smoking and other regular nicotine use
5	Anaemia.
6	Uncontrolled hypertension
7	Clinically significant out of range values of serum levels of either alanine aminotransferase (ALT), aspartate aminotransferase (AST) in the Investigator's opinion
8	Unstable/rapidly progressing renal disease or estimated glomerular filtration rate <60 mL/min (Cockcroft-Gault formula).

	Males: $\text{Creatine clearance (mL/ min)} = \frac{\text{Weight(kg) x (140 - Age)}}{\text{Serum creatine (µmol/L)}} \text{ x } 1.23$ Females: $\text{Creatine clearance (mL/ min)} = \frac{\text{Weight(kg) x (140 - Age)}}{\text{Serum creatine (µmol/L)}} \text{ x } 1.04$
9	Use of anti-coagulant treatment and other concomitant medication will be evaluated on a case-to-case basis with a general physician
10	Use of medication such as oral glucocorticoids, anti-estrogens or other medications that are known to markedly influence insulin sensitivity
11	Use of loop diuretics
12	Intake of dietary supplements except multi-vitamins and minerals
13	Alcohol consumption of > 14 drinks per week for women and > 21 drinks per week for men (1 drink = 35 cl beer, 14 cl wine or 4 cl hard liquor)
14	Known hypersensitivity to dapagliflozin or any of the excipients of the product
15	For women only - currently pregnant (confirmed with positive pregnancy test) or breast-feeding
16	Participation in another biomedical study within 1 month before the screening visit
17	Any contraindication for MRI scanning
18	Participants who do not want to be informed about unexpected medical findings, or do not wish that their physician be informed about coincidental findings, cannot participate in the study

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Chapter 7

The impact of prolonged fasting on 24h energy metabolism in healthy, lean males

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Chapter 8

General discussion and conclusion

Modern 24-hour society is characterized by frequent misalignment of the internal, circadian rhythm and the external environment, by eating and being active at times that the body is ill-prepared for it, resulting in (an increased risk of) metabolic disturbances such as obesity and type 2 diabetes. The focus of this thesis was to investigate opportunities to improve metabolic health by reinforcing the natural day-night rhythm via timed lifestyle interventions, with an emphasis on restoring the feeding-fasting cycle. This chapter provides a critical appraisal and broader perspective of the studies contained in this thesis.

To fast more furiously: The effect of a more pronounced fast on nocturnal substrate oxidation in metabolically compromised individuals

The fasting response entails multiple organs that work together with the main purpose to maintain glucose delivery to glucose-dependent cell types (i.e. neural cells, red blood cells, and renal medullary cells). In order to preserve glucose, non-glucose-dependent cell types predominantly rely on fat as a fuel during a prolonged fast. Peripheral organs further aid in the process of glucose-preservation through a diminished insulin sensitivity which lowers their glucose uptake (1, 2). The liver plays a prominent role in the fasting response since it is responsible for the majority of glucose production by means of glycogenolysis and gluconeogenesis (3). A previous study in healthy, lean volunteers, has shown that hepatic glycogen rapidly decreases during the initial 22 hours of a prolonged fast and that, at the same time, hepatic gluconeogenesis accounts for ~65% of total glucose production (4). Hepatic glycogenolysis is crucial for the fasting response, since it has been shown to trigger fat oxidation and leads to metabolic adaptations that provide alternative substrates (5). With hepatic gluconeogenesis, glucose is formed from precursors such as glucogenic amino acids and glycerol. Fasting for more than ~12 hours will stimulate the liver to produce ketone bodies which can be used by the brain, central nervous system, and the muscle as an alternative energy fuel (6). The β-oxidation of free fatty acids in white

adipose tissue forms acetyl-CoA, which can be shuttled to the liver to form ketone bodies. Alternatively, the liver also produces ketone bodies from ketogenic amino acids.

It has previously been shown that young lean males exhibit a more fasted state during the night compared to the day, characterized by a pronounced switch between daytime carbohydrate oxidation and night time fat oxidation. In addition, energy expenditure showed a peak at ~23.00 and a trough at ~04.00 (7). These responses in substrate oxidation and energy expenditure have been replicated in **chapter 7** of this thesis, when healthy lean males were provided with meals in energy balance. To assess these oscillations over 24 hours, in both studies, measurements were taken at times that were minimally affected by the acute effects of food intake and physical activity. When 24-hour oscillations in energy metabolism were assessed in older, overweight and obese males with insulin resistance, the metabolic switch between daytime carbohydrate oxidation and night time fat oxidation appeared to be lost. Hence, this group of participants did not move into a clear fasted state overnight, normally characterized by the higher fat oxidation, but remained to exhibit high nocturnal carbohydrate oxidation (8). It can be argued that fat oxidation is vital for metabolic health, since it reduces the accumulation of lipotoxic intermediates that can interfere with insulin signalling (9, 10). Indeed, a blunted response to insulin is a hallmark of the pathogenesis of type 2 diabetes and is accompanied by a reduced ability to adapt substrate availability to changes in energy demand, i.e. a reduced metabolic flexibility (11). In this thesis, it was investigated if the overnight fasted state in adults with compromised metabolic health could be restored by either increasing the time spend in fasting prior to bedtime (**chapter 4, 5**) or by use of medication (**chapter 6**).

Chapter 4 showed that acutely prolonging the overnight fasting duration from 9.5 hours to 16 hours resulted in higher overnight fat oxidation and reduced carbohydrate oxidation. This effect on substrate preference was observed in middle-aged lean individuals, as well as in middle-aged overweight/obese individuals with a

non-alcoholic fatty liver (NAFL). Although the longer fasting period did result in a higher nocturnal fat oxidation in the NAFL group, carbohydrate oxidation during the night remained relatively high, and did not normalize to the same, lower level that was found in the lean group. Despite the higher fat oxidation, hepatic glycogen depletion appeared unaltered in both groups. Thus, although acutely prolonging the fasting time did result in a higher nocturnal fat oxidation in individuals with NAFL, this fat oxidation did not reach the same level as found in the lean volunteers after a 9.5 hour fast. It can be argued that acutely prolonging the overnight fast is not sufficient to restore nocturnal fat oxidation but that repetitively prolonging the overnight fast is needed for an adaptive response of hepatic glycogen depletion to fasting, in order to normalize nocturnal fat oxidation. In order to test this hypothesis, in **chapter 5** individuals with type 2 diabetes were subjected to a 3 week time-restricted eating (TRE) intervention in which volunteers limited their habitual food intake to 10 hours during daytime. We found however, that nocturnal substrate oxidation did not appear to be affected by TRE as compared to the control condition, wherein volunteers spread their habitual diet over at least 14 hours per day. In line, hepatic glycogen content, assessed in the morning after an overnight fast, appeared unaltered with TRE. An unaltered hepatic glycogen content was observed both after a standardized fast as well as when fasting time was 14 hours with TRE compared to 10 hours in the control condition. It can be argued that the lack-of-effect of the time-restricted eating regime could be attributed to the fact that this group of individuals with type 2 diabetes is more metabolically compromised as compared to individuals with NAFL, and/or that the TRE regime was too mild to increase fat oxidation. Moreover, since the 3-week intervention period used in the TRE trial is still relatively short, it may be possible that adaptation effects will only occur over a longer period of time.

An alternative method to reinforce the overnight fast is via the type 2 diabetes medication sodium-glucose cotransporter 2 inhibitor (SGLT2i). With SGLT2i, the glucose resorption in the proximal tubules of the kidney is reduced, causing an outflow of glucose via the urine. During the night, the glucose outflow may result in

physiological mechanisms that protect against hypoglycaemia and, therefore, use of this medication could stimulate a fasting-like response. **Chapter 6** entailed a placebo-controlled trial that indeed showed that 2 weeks of the SGLT2i dapagliflozin resulted in a higher nocturnal fat oxidation in individuals with insulin resistance during the first night of their respiration chamber stay. Again, this higher fat oxidation was not accompanied by changes in hepatic glycogen content as measured in the morning. Of note, a previous placebo-controlled trial in our group showed that 5 weeks of dapagliflozin also resulted in a higher nocturnal fat oxidation in individuals with type 2 diabetes (12). Therefore, dapagliflozin may be a stronger stimulus than prolonging the fasting time to induce a pronounced overnight fasting state. However, it should be noted that in both dapagliflozin trials fat oxidation was also higher during daytime.

Thus, from these results it can be concluded that prolonging the overnight fast seems promising to restore nocturnal fat oxidation in healthy and metabolically compromised individuals without type 2 diabetes, albeit that this fasted state does not appear to be accompanied by changes in hepatic glycogen turnover. To restore nocturnal fat oxidation in individuals with type 2 diabetes a prolonged overnight fast may be insufficient and pharmacological agents such as dapagliflozin could be more effective.

Metabolic health, a matter of time? Implications of timing of physical activity, and food intake on health outcomes

In addition to the physiological mechanisms that underlie fasting, the fasting-feeding cycle is also important for the natural day-night rhythm of the body. Each cell, tissue and physiological mechanism in the body exhibits an internal, circadian rhythm which fluctuates over an approximate 24-hour time period: the circadian rhythm (13, 14). The circadian rhythm enables humans (and other organisms) to anticipate predictable changes in the environment to generate an optimal response to these changes. Conversely, changes in the environment can, in turn, synchronize the circadian rhythm

so it is attuned to the changed environment. An example of the latter is flying across time zones, when the circadian rhythm is able to adapt to the changed environmental rhythm. It should be noted here, though, that this adaptation process appears to be relatively slow and it is estimated that it takes at least a week for the internal rhythm to fully adapt to a change in time, which depends on the severity of the time difference (15). Thus, the circadian rhythm is not rigid; it responds to time cues from the environment (*Zeitgebers*) to synchronize the internal rhythm to the exact 24-hour day as is experienced on earth, which we here refer to as the day-night rhythm. Light (especially sunlight) is the most important *Zeitgeber* for the circadian rhythm and is perceived by cells in the retina which subsequently send a signal to the main clock of our body which is located in the hypothalamus: the suprachiasmatic nucleus (SCN). The SCN then synchronizes the peripheral clocks in other tissues to the light-dark cycle. However, peripheral clocks also respond to other environmental *Zeitgebers* including fasting/feeding and physical activity. Moreover, it is thought that the peripheral clocks can signal back to the SCN so that the day-night rhythm is formed by a cross-talk between the central clock and the peripheral clocks.

The importance of the interplay between internal rhythms and external time cues for metabolic health is illustrated by studies that show an increased risk of developing e.g. type 2 diabetes when rhythms are frequently being disturbed severely, as is the case in night shift work (16-18). Indeed, it has previously been shown, including in our research group (19), that simulating a night-shift in an experimental setting already deteriorates the insulin sensitivity of healthy, young volunteers (19-21). Furthermore, night shift workers frequently experience sleep disturbances (22, 23) which in turn is also associated with impaired metabolism (24, 25), reflecting the tight link between the circadian system and sleep. Importantly, already a slight disruption in the internal rhythm as experienced when going to bed late and sleeping in during free days compared to work days, termed social jetlag (26, 27), has been associated with metabolic dysfunction and obesity (26).

Considering the importance of the circadian rhythm in metabolic health, using appropriately timed lifestyle interventions to re-align the external rhythm of the environment to fit better with the internal rhythm shows promise to improve metabolism. An overview of timed lifestyle interventions is provided in **chapter 2**. From this chapter, it became clear that relatively few studies have been performed that examined the effect of the time-of-day of physical activity on obesity-related metabolic disturbances and that more research is needed, especially in humans. Nonetheless, an important study in this field showed that performing high-intensity interval training in the afternoon, as opposed to the morning, was more effective in improving the 24-hour glucose profile in males with type 2 diabetes (28). In line, a more recent observational study showed that performing most moderate-to-vigorous physical activity (MVPA) in the afternoon or evening was associated with a reduced risk of insulin resistance in the general population as compared to evenly spreading MVPA over the day (29). In **chapter 3** of this thesis, it was examined if the previously found association between MVPA and insulin resistance may be explained by a better sleep quality when performing most MVPA in the afternoon/evening. As mentioned earlier, sleep is an important determinant of metabolic health and is tightly connected to the circadian rhythm. However, **chapter 3** did not show strong evidence for a relationship between time-of-day of MVPA and sleep quality. Therefore, the previously found association between afternoon and evening MVPA and insulin resistance appears to be independent of changes in sleep quality. Importantly, MVPA reflects all physical activity performed at moderate-to-vigorous intensity, whereas exercise entails a subset of physical activity that is planned and structured and usually is performed for a longer period of time and is often of higher intensity (30). Thus, exercise can elicit physiologic adaptations that are not necessarily elicited by MVPA, and it remains possible that time-of-day of exercise is important for sleep quality.

Food intake is another *Zeitgeber* for the circadian rhythm, and considering the effects of food intake on multiple metabolic organs, food intake is probably the most important time cue for metabolism (31). Generally, food intake occurs during the

active period, which for diurnal animals, such as humans, is at daytime. However, an important observational study from 2015 showed that the median time window of food intake spanned almost 15 hours in the general population, indicating that food intake is not limited to only daytime for most individuals (32). In this thesis, it was posed that this wide eating time window is detrimental for metabolic health, since it hampers entering a profound overnight fasting state and consequently results in a limited utilization of energy stores. It was hypothesized that prolonging the overnight fast would result in a higher utilization of energy stores which would stimulate replenishment of the energy stores with the first meal after the overnight fast. Simultaneously, prolonging the overnight fast would fit better with the human internal rhythm of daytime eating and fasting at night. In **chapter 4**, it was shown that acutely prolonging the overnight fasting period from 9.5 hours to 16 hours had only limited effects on postprandial metabolism in response to a meal test in middle-aged lean volunteers and in middle-aged overweight/obese volunteers with NAFL. In **chapter 5**, prolonging the overnight fast to ≥ 14 hours for 3 weeks did result in an improved glucose homeostasis in volunteers with type 2 diabetes, as reflected by more time spent in the normoglycemic range, lower fasting glucose levels, and lower 24-hour glucose levels as compared to the control condition, where the overnight fast was limited to ≤ 10 hours per day. However, these changes did not appear to be accompanied by alterations in insulin sensitivity, hepatic glycogen turnover, or 24-hour substrate metabolism. The improved glucose homeostasis may be explained by the observation that volunteers lost significantly more weight with TRE as compared to their control condition, which has also been reported previously (see **chapter 2** for an overview). Nevertheless, a previous highly-controlled randomized cross-over trial in men with prediabetes showed an improved insulin sensitivity with an early TRE regime which was not accompanied by changes in weight (33). Importantly, the study in **chapter 5** also showed that the improvement in glucose levels could be mainly attributed to lower glucose levels during the night and an (expected) earlier decrease in glucose levels occurring at ~18.00 after which participants were not allowed to eat

anymore. Furthermore, the weight loss difference between TRE and the control condition was only modest (0.7 kg). Combined, these results suggest that TRE also results in metabolic benefits which could be independent of weight loss. The underlying mechanisms are however still unclear but may contain a circadian component.

As mentioned earlier, the rhythmicity in 24-hour substrate oxidation may also be influenced by the drug dapagliflozin, which is an SGLT2i. **Chapter 6** showed that dapagliflozin treatment resulted in lower 24-hour glucose levels and an improved skeletal muscle mitochondrial capacity. The glucose outflow induced with dapagliflozin occurs over 24-hour and is higher during daytime as compared to the night. Therefore, it is likely that the mechanisms underlying the effect of SGLT2i are comparable to calorie restriction. Calorie restriction-induced weight loss has been shown to improve metabolic flexibility in obese glucose-intolerant men (34). As such, SGLT2i treatment may, on the long term, also be able to (partially) restore the metabolic switch between daytime glucose oxidation and night time fat oxidation by its favourable effects on weight, but this remains to be investigated.

Stay tuned: Looking into food intake as modulator of metabolic day-night rhythms

As mentioned previously, external *Zeitgebers* have the ability to shift the rhythmicity of internal, metabolic processes. Therefore, in this thesis the effects of food intake on metabolic rhythmicity were also assessed. In **chapter 5** of this thesis, the rhythmicity of glucose levels was shifted with TRE. Thus, with TRE, glucose levels started decreasing earlier, i.e. at ~18.00 (at the end of the eating time window), as compared to the control condition, which was the result of the last meal that was consumed earlier in the day.

Furthermore, **chapter 7** of this thesis examined the changes in the day-night rhythm of energy metabolism in the total absence of food intake, i.e. during a 60-hour fast.

Since food intake elicits various metabolic processes and as such is an important *Zeitgeber*, it may also affect the rhythmicity in substrate oxidation and energy expenditure. As expected, fat oxidation was higher during the fast, and increased progressively with fasting duration whereas carbohydrate oxidation decreased to a minimum. These adaptations in substrate oxidation caused that the day-night rhythmicity in substrate oxidation differed from the rhythm that was found when participants received meals in energy balance. More specifically, there was a loss of day-night rhythmicity in substrate oxidation when fasted. In contrast, the rhythm in energy expenditure was similar with fasting as compared to the fed condition, both when measured after 1 day of fasting as well as after 2 days of fasting. Combined, these findings indicate that especially the rhythm in substrate oxidation is driven by food intake.

The future comes with time

At present, knowledge about re-aligning the environmental rhythm with the internal rhythm is limited, and therefore there is much to explore in this area of research. Below are two directions outlined which are deemed promising for future studies.

TRE: to breakfast or not to breakfast

Time-restricted eating is a promising lifestyle intervention to improve insulin sensitivity and glucose homeostasis, that may be more easy to adhere to than more conventional dietary interventions. Importantly, in contrast to more common dietary interventions, TRE does not require any calorie counting or dietary recommendations, although TRE does require the ability to refrain from food intake for a prolonged period of time. Currently no consensus exists on the most adequate time window for TRE. Hence, most previous studies have focused on early TRE (eTRE), whereby breakfast is consumed in the morning and the period of fasting commences after the

last meal is consumed in the afternoon/ early evening (see also **chapters 2** and **5** of this thesis). Possible mechanisms underlying the metabolic benefits of eTRE may – next to the more pronounced fed-fasting cycle as studied in this thesis - also include a higher diet-induced thermogenesis in the morning vs the evening, or a higher glucose tolerance in the morning (studies described in more detail in **chapter 2**). In addition, evidence from human observational and intervention studies suggest that consuming the majority of energy early in the day is an effective weight loss strategy (35-40). In line, it has also been suggested that eating early in the day promotes a lower energy intake in individuals with type 2 diabetes as compared to eating later in the day (41) which may explain some of the results on weight loss effectiveness. Importantly, randomized controlled trials also showed a higher fat oxidation (42) and a higher daytime energy expenditure (43) with eating breakfast compared to skipping breakfast, when energy intake and macronutrient composition are similar for early vs late eating. The latter suggests that eTRE also has beneficial metabolic effects that are independent of weight loss.

Nevertheless, not all studies support the hypothesis that eating breakfast has superior effects over skipping breakfast. For example, recently, a 12-month randomized controlled weight loss trial was published that showed that metabolically healthy obese individuals did not lose more weight when they consumed a low-calorie diet between 08.00-16.00 as compared to consuming the same diet without constrains on timing of intake (44). Similarly, when the effects of a 7-day eTRE (08.00-17.00) regime were compared with a delayed TRE (12.00-21.00) in males with prediabetes, the eTRE regime only proved to be superior in improving fasting glucose whereas the improvement in glucose tolerance was similar between the two regimes (45).

The difficulty in interpreting studies that investigate early vs late eating regimes is the plethora of factors involved that can influence the metabolic response. In this thesis, it has been shown that fasting duration can modulate metabolism and therefore fasting duration needs to be standardized when comparing early vs late TRE

regimes. In addition, literature suggests that not merely the timing of food intake is important, but also the macronutrient composition of the food that is ingested matters (46, 47). These are important issues to address in future studies since skipping breakfast may be easier to implement in daily life as compared to skipping late-night eating, and thus a later TRE protocol could be more feasible to implement as long-term lifestyle intervention. Finally, it may be possible that individuals have differential metabolic responses to the same TRE regime, which will be discussed next.

Tailoring timed interventions to an individual's metabolic make-up and lifestyle

It may be possible that the optimal time window for eating and being active differs per person and may be attenuated by the preferred time of day at which a person is active, which is referred to as chronotype. However, there are still many knowledge gaps regarding the tailoring of the timing of lifestyle interventions to a person's chronotype. Chronotype is partially determined genetically; the protein components involved in the molecular clock machinery can differ between individuals resulting in a differential adaptation to the same light-dark cycle (48). In addition, the strength of the environmental time cues also plays a role in the entrainment of the internal clock. For example, exchanging electrical light for natural daylight results in an earlier chronotype; the stronger daylight exposure causes humans to go to bed and to wake up earlier (49). In addition, chronotype varies per season since the circadian rhythm adapts to differences in the light-dark cycle (48). Thus, it appears difficult to unravel a person's chronotype and adapt lifestyle interventions accordingly.

Furthermore, not only chronotype may attenuate the response to a timed intervention, also the metabolic response to an intervention itself can differ per person. In nutritional research, it has been shown that a high inter-individual variability exists in response to identical test meals (50). Tailoring the optimal diet to a person's metabolic phenotype remains challenging, due to the many parameters involved in the metabolic response, including dietary habits, anthropometrics and gut microbiota

(50). Similarly, a high inter-individual variability has also been reported for post-training adaptations in response to aerobic- as well as resistance exercise interventions (51). As such, some individuals respond very strong to an exercise intervention whereas others do not appear to respond at all (so-called non-responders). It has been argued that exercise modality (aerobic vs resistance) plays an important role in this variability, since individuals can be responders for aerobic but non-responders for resistance exercise (51).

Thus, for future studies it will be interesting to direct the timing and type of interventions to the chronotype as well as the metabolic phenotype of the individual. However, large-scale studies will be needed to develop algorithms that can be used in order to achieve this goal.

Summary

This thesis focussed on improving metabolic health by reinforcing the natural day-night rhythm via timed lifestyle interventions, with a focus on restoring the feeding-fasting cycle. **Chapter 2** showed that the current body of evidence for timed interventions is limited and that a need for timed human intervention studies exists. Specifically, the effect of timing of physical activity on metabolic outcomes is a relatively understudied field in humans. Results from an observational study suggested that performing most moderate-to-vigorous physical activity (MVPA) in the afternoon and evening was associated with a reduced insulin resistance compared to evenly distributing MVPA over the day. Physical activity is an important time cue for the circadian rhythm, which in turn regulates sleep. Importantly, sleep disturbances are also implicated in the development of metabolic disturbance. Therefore, we examined in **chapter 3** whether performing most MVPA in the afternoon and evening was associated with a higher quality of sleep. We found no associations between the time window at which MVPA was performed and most sleep characteristics. As such, the

previously found association between afternoon and -evening MVPA and insulin resistance appears to be independent of changes in sleep quality.

In addition to physical activity, food intake plays a key role in attuning the circadian rhythm of metabolic processes to the 24h environmental rhythm. Therefore, **chapters 4** and **5** examined whether restoring the feeding-fasting cycle by prolonging the fasting duration prior to bedtime, i.e. having an earlier dinner, resulted in a more pronounced overnight fast and concomitant metabolic benefits. **Chapter 4** showed that acutely prolonging the fasting duration from 9.5 hours to 16 hours resulted in an improvement in fat oxidation in healthy individuals as well as in individuals with a non-alcoholic fatty liver. The improvement in fat oxidation was, however, not accompanied by changes in hepatic glycogen content or changes in the metabolic response to a breakfast meal. In **chapter 5**, we showed that limiting food intake to a 10-hour window (with the last meal at 18.00 the latest) for a period of 3 weeks did not result in changes in energy metabolism or hepatic glycogen content in individuals with type 2 diabetes compared to spreading food intake over at least 14 hours per day (control). Nevertheless, glucose homeostasis was improved with TRE vs control, reflected by more time spent in the normal glucose range and lower fasting glucose and 24h glucose levels (**chapter 6**). **Chapter 6** examined if a more pronounced nocturnal fast in could also be induced with the type 2 diabetes drug dapagliflozin, which results in a higher outflow of urinary glucose. Indeed, this 2-week placebo controlled trial showed a higher nocturnal fat oxidation in individuals with insulin resistance, which was not accompanied by changes in hepatic glycogen content. Dapagliflozin treatment also resulted in lower 24-hour glucose levels and an improved skeletal muscle mitochondrial capacity compared to placebo.

Finally, **chapter 7** examined the impact of a 60h fast on 24h energy metabolism in healthy, lean males. Fat oxidation rapidly increased during the fast, and kept increasing, whereas carbohydrate oxidation decreased to a minimum. Although there was a loss-of-rhythmicity for substrate oxidation in the fasted vs fed condition, the

rhythm in energy expenditure appeared to be unaffected. These results suggests that the rhythm in energy expenditure exists independent of food intake whereas the rhythm in substrate oxidation is driven by food intake.

In sum, this thesis shows that the timing of lifestyle interventions matters for metabolic health and that more human intervention studies are needed to gain a better understanding of mechanisms underlying timed lifestyle interventions.

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Addendum

Summary

Virtually all cells, tissues and physiological processes in the human body exhibit an internal rhythm that can be entrained to the environmental rhythm experienced on earth by (sun)light exposure, food intake, and physical activity (so-called time cues or *Zeitgebers*). The biological, circadian rhythm takes approximately 24 hours to complete and is present even in absence of time cues, whereas the day-night rhythm represents the rhythm that has been entrained to the environmental 24h rhythm. The entrainment of the internal rhythm allows the human body to respond efficiently to predictable homeostatic challenges, such as eating and periods of sleep. In turn, the internal rhythm can be adjusted by changes in the environmental rhythm via *Zeitgebers*. As such, when travelling across time zones, humans are able to adapt their internal rhythm to a time zone that is different from their home country. Similarly, humans can adapt to altered day-night rhythms, for example when performing shift work. However, in recent decades, it has become evident that too frequently challenging the internal rhythm by eating and being active at irregular times, such as with night-shift work, has detrimental consequences for metabolic health. Disturbances of the internal rhythm can have a direct, negative effect on metabolic health, but can also indirectly deteriorate metabolic health via a disturbed sleep. Although it is clear that frequently disturbing the internal rhythm results in impaired metabolic health, it is thus far unclear if metabolic health could benefit from lifestyle interventions that are aligned with the internal rhythm. Therefore, in this thesis, we sought to investigate if re-aligning the timing of physical activity and food intake with the natural biological rhythm had potential to improve metabolic health. The emphasis of this thesis was to restore the 24-hour rhythm in feeding and fasting, which becomes disturbed when eating at irregular times. Both observational- as well as clinical studies were performed to address the aim of this thesis.

On a physiological level, circadian rhythms are shaped by an interplay between the central clock (or master pacemaker), which is located in the suprachiasmatic nucleus (SCN) of the posterior hypothalamus and by auxiliary clocks located in peripheral tissues. The most important *Zeitgeber* for the clocks is (sun)light, that is picked up by retinal cells, which subsequently send a signal to the SCN. Ultimately, this process results in the synchronization of all body clocks to sunlight. In addition to light, peripheral clocks can also be attuned by food intake and exercise, and can signal back to the SCN. **Chapter 2** of this thesis provides a theoretical framework on the working mechanisms of the circadian rhythm, as well as an overview of studies examining rhythmicity in human metabolism. Importantly, **chapter 2** also provides an assembly on human intervention studies that have been performed on the effect of timed lifestyle interventions on obesity-related metabolic diseases. From this chapter, it became apparent that it is currently unclear whether an optimal time window to perform physical activity exists to gain most metabolic benefits. Recently, a cross-sectional study showed that people who perform most moderate-to-vigorous physical activity (MVPA) in the afternoon and evening had a higher insulin sensitivity compared to those that evenly distributed their MVPA over the day. It could be hypothesized that performing most activity in the afternoon or evening has different effects on sleep quality compared to activity performed in the morning. Thus, the results from this study may be explained by differences in sleep quality, since sleep is related to metabolic health and regulated by the circadian rhythm. To address this hypothesis, in **chapter 3**, a cross-sectional data analysis was performed to investigate if performing most MVPA in the afternoon and evening was also associated with optimal sleep quality. However, this study did not show associations between the time window in which MVPA was performed and most of the measured sleep characteristics. These results therefore imply that the previously found association between afternoon/evening MVPA and insulin sensitivity was likely not influenced by changes in sleep with afternoon/evening MVPA.

The subsequent chapters of this thesis were aimed at understanding the effects of a longer fasting duration, by advancing the last meal of the day, on metabolic health. These studies were conducted since a previous study showed that most people have an eating time window that spans at least 14 hours and that reducing this eating time window, thereby prolonging fasting time, can result in weight loss. Importantly, the weight loss occurred without any dietary restrictions. Moreover, a subsequent study in males at risk of type 2 diabetes showed that limiting food intake to a more narrow time window, thereby prolonging the overnight fast, results in an increased insulin sensitivity, even without weight loss. Nevertheless, the mechanisms underlying the benefits of limiting food intake to a narrow time window remained unclear. In this thesis, it was hypothesized that when the duration of the daily overnight fast is prolonged, this results in an increased utilization of nutrient stores, such as fat stores and hepatic glycogen, which could improve the uptake of nutrients with the first (breakfast) meal of the subsequent day. Thus, **chapter 4** showed that acutely prolonging the fasting duration from 9.5 hrs to 16 hrs was successful in increasing nocturnal fat oxidation in middle-aged healthy individuals, as well as in middle-aged individuals with a non-alcoholic fatty liver (NAFL). A prolonged fasting duration was, however, not accompanied by alterations in the metabolic response to a standardized breakfast meal or on hepatic glycogen utilization, neither in the healthy nor in the NAFL group. It could be argued that acutely prolonging the overnight fast is too short to improve metabolic health and that repeatedly prolonging the overnight fast would result in more metabolic benefits. Therefore, the effects of a 3-week time restricted eating (TRE) regime were investigated in individuals with type 2 diabetes (**chapter 5**). In contrast to the acute study, prolonging the fasting duration from 10 hrs (control) to 14 hrs (TRE) for a period of 3 weeks did not result in a higher nocturnal fat oxidation. Also levels of hepatic glycogen were not significantly lower after TRE in individuals with type 2 diabetes. Nevertheless, the TRE intervention did result in improvements in glucose homeostasis, which was reflected by a reduction in 24h glucose and fasting glucose levels and more time spent in the normal glucose range. Combined, these

results suggest that prolonging the fasting duration is a promising strategy to improve metabolic health. However, it may be possible that stronger effects are obtained when a more pronounced overnight fasting response is elicited by medication. The type 2 diabetes medication sodium-glucose cotransporter 2 inhibitor (SGLT2i) is promising in this respect, since this medication blocks the resorption of glucose in the proximal renal tubules, which results in a higher urinary glucose excretion. During the night, this medication could lead to a more pronounced fasted state, which subsequently could trigger mechanisms that protect against hypoglycaemia. In **chapter 6**, we showed that two weeks of the SGLT2i dapagliflozin indeed resulted in higher nocturnal fat oxidation, which was accompanied by lower 24-hour glucose levels, higher 24-hour FFA levels, and lower nocturnal levels of beta-hydroxybutyrate in adults with insulin resistance when compared to the placebo. In addition, maximal mitochondrial oxidative capacity was also higher with dapagliflozin. No changes were found in hepatic glycogen and -lipid content.

Finally, since food intake plays a key role in the day-night rhythm of metabolic processes, **chapter 7** of this thesis examined the effect of a 60 hour fast on the day-night rhythmicity in energy metabolism in healthy lean males. In addition, this study visualised the changes in energy metabolism that occur over time when fasted for a longer period of time. This study showed that fasting resulted in changes in rhythmicity of substrate oxidation, whereas the rhythm in energy expenditure remained unaffected, suggesting that the rhythm of energy metabolism is not driven by food intake. Further, the switch to a relatively higher fat oxidation occurred early on during the 60-hour fast and kept gradually increasing over the whole fasting period; nevertheless, carbohydrate oxidation was still present, albeit in minimal quantities.

In sum, this thesis shows that re-aligning the timing of exercise and food intake with the natural day-night rhythm, as well as restoring the rhythm of the fasting-feeding cycle by inducing a more pronounced overnight fast, can improve metabolic health. Importantly, this thesis also emphasizes the need for more human intervention

studies to find the optimal time-of-day to eat and to be active. Furthermore, more mechanistical studies are needed to gain a better understanding of the processes underlying the metabolic health benefits of timed lifestyle interventions.

Samenvatting (NL)

Nagenoeg alle cellen, weefsels en fysiologische processen in het menselijk lichaam vertonen een intern ritme dat zich kan aanpassen aan het ritme van de omgeving door middel van (zon)licht, voedselinname en lichaamsbeweging (zogenoemde tijdsaanwijzingen of *Zeitgebers*). Het biologische, circadiaanse ritme heeft een lengte van ongeveer 24 uur en is zelfs aanwezig wanneer er geen tijdsaanwijzingen zijn, terwijl het dag-nacht ritme verwijst naar het ritme dat zich heeft aangepast aan het 24-uurs ritme van de omgeving. De eigenschap van het interne ritme om zich aan te passen aan de omgeving heeft als voordeel dat het menselijk lichaam efficiënt kan reageren op voorspelbare verstoringen van de homeostase, zoals gebeurt bij voedselinname en periodes van slaap. Daarnaast kan het interne ritme door middel van *Zeitgebers* afgestemd worden op veranderingen in het ritme van de omgeving. Een voorbeeld van een verandering in het ritme van de omgeving is het reizen door tijdzones, wanneer mensen in staat zijn om hun interne ritme aan te passen aan het omgevingsritme dat verschillend is van hun thuisland. Een ander voorbeeld is het draaien van nachtdiensten, wanneer mensen actief zijn op tijden dat ze normaal zouden liggen te slapen. Onderzoek in de afgelopen decennia heeft overtuigend aangetoond dat er nadelige metabole gezondheidseffecten kunnen optreden wanneer het interne ritme te vaak wordt verstoord door op onregelmatige tijden actief te zijn en te eten. Dit is met name het geval met het draaien van nachtdiensten. Verstoringen van het interne ritme kunnen een direct, negatief effect hebben op de metabole gezondheid, maar kan ook indirect negatieve gevolgen hebben voor de metabole gezondheid door een verstoorde slaap. Hoewel het duidelijk is dat het vaak verstoren van het interne ritme een verslechterde metabole gezondheid als gevolg kan hebben, was het tot nu toe onduidelijk of de metabole gezondheid kan verbeteren door middel van leefstijlinterventies die afgestemd zijn op het interne ritme. Daarom was het doel van dit proefschrift om te onderzoeken of het afstemmen van de timing van lichaamsbeweging en voedselinname op het natuurlijke biologische ritme

mogelijkheden biedt om de metabole gezondheid te verbeteren. Dit proefschrift focuste zich met name op het herstellen van het 24-uurs ritme van eten en vasten, wat verstoord raakt wanneer er op onregelmatige tijden wordt gegeten. Zowel observationele- als klinische studies zijn uitgevoerd om het doel van dit proefschrift te bereiken.

Op fysiologisch niveau worden interne ritmes gevormd door een samenspel tussen de centrale klok (of hoofdpacemaker), die zich in de suprachiasmatische nucleus (SCN) van de achterste hypothalamus bevindt, en door perifere klokken in de weefsels van de rest van het lichaam. De belangrijkste *Zeitgeber* voor de klokken is (zon)licht, dat wordt opgevangen door cellen in het netvlies, die vervolgens een signaal naar de SCN sturen. Dit proces zorgt er uiteindelijk voor dat alle lichaamsklokken synchroon lopen met zonlicht. Naast licht kunnen perifere klokken ook worden afgestemd op voedselinname en lichaamsbeweging, en kunnen ze een signaal terugsturen naar de SCN. Hoofdstuk 2 van dit proefschrift geeft een theoretische achtergrond van de werkingsmechanismen van het circadiaanse ritme, evenals een overzicht van onderzoeken naar ritmiek in de menselijke stofwisseling. Ook geeft hoofdstuk 2 een overzicht van humane interventiestudies die over het effect gaan van getimede leefstijlinterventies op stofwisselingsziekten die in verband staan met obesitas. Uit dit hoofdstuk kwam er naar voren dat het op dit moment onduidelijk is of er een optimaal tijdvenster bestaat om lichamelijk actief te zijn waardoor de meeste metabole voordelen worden behaald. Onlangs toonde een dwarsdoorsnede onderzoek aan dat mensen die 's middags en' s avonds de meeste matig-tot-intensief inspannende lichaamsbeweging (MVPA) uitvoeren een hogere insulinegevoeligheid hadden in vergelijking met degenen die hun MVPA gelijkmatig over de dag verdeelden. Deze resultaten zouden er misschien op kunnen wijzen dat meer lichaamsbeweging in de middag of avond een ander effect heeft op de slaapkwaliteit dan lichaamsbeweging in de ochtend. Wanneer we deze beredenering volgen dan zouden de resultaten van

voorgaand onderzoek wellicht kunnen worden verklaard door verschillen in slaapkwaliteit, aangezien slaap belangrijk is voor de metabole gezondheid en wordt beïnvloed door het circadiaanse ritme. Om deze hypothese te testen werd in hoofdstuk 3 een dwarsdoorsnede data-analyse uitgevoerd om te onderzoeken of het uitvoeren van de meeste MVPA in de middag en avond ook geassocieerd was met een optimale slaapkwaliteit. Deze studie liet echter geen verband zien tussen het tijdvenster waarin MVPA werd uitgevoerd en de meeste gemeten slaapkenmerken. Deze resultaten kunnen er daarom op wijzen dat het eerder gevonden verband tussen middag/avond MVPA en insulinegevoeligheid waarschijnlijk niet werd beïnvloed door veranderingen in slaap met middag/avond MVPA.

De hoofdstukken van dit proefschrift die volgde waren er op gericht om de effecten van het verlengen van de vastenperiode op de metabole gezondheid te begrijpen door middel van het vervroegen van de laatste maaltijd van de dag. Deze studies werden uitgevoerd omdat een eerdere studie aantoonde dat de meeste mensen hun dagelijkse maaltijden over een tijdsvenster van minstens 14 uur spreiden en dat het verkorten van dit eettijdvenster, waardoor de vastentijd wordt verlengd, kan leiden tot gewichtsverlies. Dit was met name interessant omdat het gewichtsverlies zonder dieetadviezen plaatsvond. Bovendien toonde een latere studie aan dat de insulinegevoeligheid van mannen met een hoger risico op diabetes type 2 kan worden verhoogd door de voedselinname tot een korte tijdsperiode te beperken waarmee de vastenperiode in de nacht wordt verlengd. De hogere insuline gevoeligheid ging niet samen met gewichtsverlies. Deze voorgaande studies gaven slechts weinig inzicht in de fysiologische processen die verantwoordelijk zijn voor de metabole voordelen van het beperken van de voedselinname tot een specifiek tijdsbestek. In dit proefschrift werd de hypothese getest dat de energievoorraden, zoals vetreserves en leverglycogeen, meer worden aangesproken wanneer de vastenperiode wordt verlengd wat de opname van voedingstoffen tijdens de eerste maaltijd van de volgende dag (ontbijt) zou kunnen verbeteren. Hoofdstuk 4 liet zien dat het eenmalig verlengen van de vastenduur van 9,5 uur naar 16 uur de nachtelijke vetoxidatie bij

gezonde personen van middelbare leeftijd en bij personen van middelbare leeftijd met een niet-alcoholische leververvetting (NAFL) kan verhogen. In zowel de gezonde proefpersonen als in de proefpersonen met NAFL ging de langere vastenperiode niet samen met veranderingen in de metabole reactie op een gestandaardiseerde ontbijtmaaltijd of met veranderingen in leverglycogeen. Het zou kunnen dat het eenmalig verlengen van de nachtelijke vastenperiode te kort is om de metabole gezondheid te verbeteren. Om te onderzoeken of het herhaaldelijk verlengen van de vastenperiode meer metabole gezondheidsvoordelen opleverde werden de effecten van een tijdsgebonden eten (TRE) regime van 3 weken onderzocht bij personen met diabetes type 2 (hoofdstuk 5). In tegenstelling tot de studie waarbij de vastenperiode eenmalig werd verlengd, leidde het verlengen van de vastenduur van 10 uur (controle) naar 14 uur (TRE) voor een periode van 3 weken niet tot een hogere nachtelijke vetoxidatie. Ook leverglycogeen was niet significant lager na TRE bij personen met diabetes type 2. De TRE leefstijlaanpassing leidde wel tot verbeteringen in de glucosehomeostase, wat gekenmerkt werd door een verlaging van de 24-uurs glucose waardes en de nuchtere glucose waardes. Ook was de periode waarin de glucose spiegels binnen de gewenste waardes lagen langer met TRE ten opzichte van de controle conditie. Samengevat laten deze resultaten zien dat het verlengen van de vastenperiode een veelbelovende strategie is om de metabole gezondheid te verbeteren. Het zou echter kunnen zijn dat een sterkere nachtelijke vastenreactie tot stand kan worden gebracht met behulp van medicatie.

In dit opzicht is het zogenoemde sodium-glucose cotransporter 2-remmer (SGLT2i) medicijn dat wordt gebruikt bij mensen met type 2 diabetes veelbelovend, aangezien het gebruik ervan ervoor zorgt dat er meer glucose in de urine terecht komt. Dit komt omdat SGLT2i de resorptie van glucose in de proximale tubuli van de nier blokkeert. Dit medicijn kan ervoor zorgen dat de vastenreactie tijdens de nacht wordt versterkt, wat ervoor zorgt dat er mechanismes worden geactiveerd die bescherming bieden

tegen hypoglykemie. In hoofdstuk 6 laten we zien dat volwassenen met insulineresistentie na een twee weken durende behandeling met de SGLT2i dapagliflozine een hogere nachtelijke vetoxidatie hadden dan na de placebo-behandeling. Deze bevinding ging gepaard met lagere 24-uurs glucosewaarden, hogere 24-uurs vrije vetzuurwaarden en lagere niveaus van beta-hydroxybutyraat (alleen tijdens de nacht gemeten). Bovendien was de maximale mitochondriale oxidatieve capaciteit ook hoger na de behandeling met dapagliflozine. Er werden geen veranderingen gevonden in de hoeveelheid glycogeen en -vetten in de lever.

Tot slot onderzochten we in hoofdstuk 7 van dit proefschrift het effect van 60 uur vasten op het dag-nachtritme in het energiemetabolisme van gezonde slanke mannen, aangezien voedselinname een sleutelrol speelt in het dag-nachtritme van metabole processen. Daarnaast bracht deze studie de veranderingen in energiemetabolisme in kaart die in de loop der tijd optreden tijdens een langere periode van vasten. Deze studie toonde aan dat vasten het ritme van de substraatoxidatie veranderde, terwijl het ritme in energieverbruik onaangetast bleef. Dit doet vermoeden dat het ritme van energieverbruik niet wordt beïnvloed door voedselinname. Verder vond de verandering naar een relatief hogere vetoxidatie al vroeg plaats tijdens de 60-urige vastenperiode en vetoxidatie bleef geleidelijk toenemen tijdens de gehele vastenperiode. Ondanks de toename in vetoxidatie was er nog steeds koolhydraatoxidatie aanwezig tijdens de vastenperiode, zij het in minimale hoeveelheden.

Uit dit proefschrift kunnen we opmaken dat het aanpassen van de timing van lichaamsbeweging en voedselinname aan het natuurlijke dag-nachtritme de metabole gezondheid kan verbeteren. Op een vergelijkbare manier kan het herstellen van het ritme van de vasten-voedingscyclus door een sterkere vastenreactie tijdens de nacht ook de metabole gezondheid verbeteren. Daarnaast benadrukt dit proefschrift het belang van meer humane interventiestudies om uit te zoeken wat het beste tijdstip van de dag is om te eten en actief te zijn. Bovendien zijn meer mechanistische studies

nodig om de processen die de metabole gezondheidsvoordelen van getimede leefstijlinterventies veroorzaken beter te kunnen begrijpen.

Impact

What is the main objective of the thesis, and what are the most important results and conclusions?

The main objective of this thesis is to examine if re-aligning the timing of physical activity and food intake with the natural biological rhythm, as well as reinforcing the eating-fasting cycle, has the potential to improve metabolic health. **Chapter 2** explains the working mechanisms of the biological rhythm and provides an assembly on current evidence on the potential of timed lifestyle interventions to improve metabolic health. Although the current body of evidence on beneficial metabolic effects of timed interventions is promising, there is still only a limited amount of human intervention studies that addresses timed interventions, and also the understanding of underlying mechanisms is lacking. More specifically, from this review it became clear that there is little knowledge in humans about the health effects of performing physical activity at a specific time-of-day. Therefore, **chapter 3** assessed the relationship between the amount and timing of moderate-to-vigorous physical activity (MVPA) on subjective sleep quality and -duration. Sleep was chosen as outcome variable since it is both related to metabolic health and regulated by the internal biological rhythm. This study found that performing a higher amount of MVPA was associated with a lower odds of fatigue-related dysfunction during daytime. Additionally, performing most MVPA in the morning was associated with fewer sleep disturbances compared to similarly distributing MVPA over the day. The subsequent **chapters 4** and **5** were devoted to investigate the effects of advancing dinner time and thereby prolonging the overnight fast on metabolic health. **Chapter 4** shows that acutely prolonging the overnight fast from 9.5 hours to 16 hours resulted in a higher overnight fat utilization in both middle-aged volunteers with a non-alcoholic fatty liver (NAFL) as well as in middle-aged healthy lean controls. However, even during the 16-hour fast, nocturnal fat utilization did not reach the same level in the volunteers with NAFL compared to that observed

in the control volunteers, even when the control volunteers were fasted for a shorter amount of time. Thus, although prolonged fasting improves fat oxidation in adults with NAFL, it does not result in a normalization of fat oxidation. Furthermore, in both groups, hepatic glycogen utilization did not change in response to the longer time fasting. In **chapter 5** the metabolic efficacy of a time restricted eating (TRE) regime was tested in adults with type 2 diabetes. The main finding of this study was that limiting food intake to a 10-hour daily time window for a period of 3 weeks resulted in lower fasting- and 24-hour glucose levels as compared to the control condition, in which volunteers spread their habitual food intake over at least 14 hours per day. In addition, volunteers spent significantly more time in the normal glucose range with TRE. Importantly, it was found that eating within 10 hours during daytime was safe and feasible to follow for adults with type 2 diabetes.

In **chapter 6**, a pharmacological approach was used to create a more pronounced fasted state by studying the diabetes medication dapagliflozin, a sodium-glucose cotransporter 2 inhibitor (SGLT2i), in adults with insulin resistance. This medication blocks the resorption of glucose in the proximal renal tubules resulting in a higher urinary glucose excretion. Two weeks of dapagliflozin resulted in lower 24-hour glucose levels, higher 24-hour FFA levels, and lower nocturnal levels of beta-hydroxybutyrate as compared to the placebo. In addition, maximal mitochondrial oxidative capacity was higher with dapagliflozin. No changes were found in hepatic glycogen and -lipid content.

To gain more insight into the physiological mechanisms that underlie the fasting response, **chapter 7** looks into the acute effects of a 60-hour fast versus fed condition on energy metabolism and -rhythmicity in healthy young male volunteers. This study showed a rapid switch to fat oxidation during the prolonged fast, and fat oxidation kept increasing gradually during the 60h fast. Carbohydrate oxidation decreased to a minimum during the fast, although it scarcely became zero. Inspection of the day-night rhythm of energy metabolism showed that particularly the rhythms in substrate

oxidation were driven by food intake, whereas the rhythm in energy expenditure remained unaltered with fasting.

What is the contribution of the research results to science and society?

For the last couple of centuries, society is struggling with an increasing burden of chronic diseases, including obesity and type 2 diabetes. According to the WHO, global deaths that could be attributed to diabetes increased with a staggeringly 70% from the year 2000 to 2019. In addition, during the recent COVID-19-pandemic it became painfully clear that the societal burden of chronic diseases does not only consist of increasing healthcare costs. During this pandemic, people with a disturbed metabolism were inflicted the most, and hospitals were overflowing. This illustrates that the current medical facilities are poorly equipped for the current disease load and emphasizes the need for improvement of metabolic health.

Although human lifestyle intervention studies generally have a limited effect on societal health, they can have an impact on health at the individual level. Importantly, human intervention studies are capable of unravelling cause-and-effect relationships which can be used as a foundation for policy makers to adjust health care recommendations or to implement measures that are able to improve metabolic health on a large scale. Poor diet and low levels of physical activity have traditionally been viewed as the most important lifestyle behaviours that underlie the development of obesity and type 2 diabetes. Studies in this thesis add to the change in this vision by highlighting that the timing of eating and being active also impacts metabolic health. Results of this thesis build on the existing body of evidence that indicates that eating and being active at the wrong time-of-day, as with night-shift work, is related to poor metabolic outcomes such as weight gain and an increased risk of developing type 2 diabetes.

Not only night-shift work disturbs the natural day-night rhythm, also flying across continents (experienced as jetlag) and going to bed late and sleeping in during the weekend as compared to work days (societal jetlag) disturbs the internal rhythm. Moreover, current 24-hour society facilitates eating around the clock, with omnipresent food availability, and thereby discourages the abstinence of food intake resulting in a disturbed feeding-fasting rhythm. Studies have indeed shown that most people spread their food intake over the larger part of the day. Consequently, people are still eating in the evening and night, when the body is less prepared to handle the nutrient load. Results in this thesis emphasize the importance of a pronounced fasting state during the night for metabolic health.

A large part of the society also suffers from sleep problems. This is concerning, since sleep affects both mental- as well as physical health, including metabolic health. Indeed, a good quality of sleep is important for the prevention of type 2 diabetes but has been long overlooked in most previous metabolic studies. Importantly, the preferred treatment for sleep problems is behavioural intervention, since sleep medication generally has negative side effects and a short-lasting effectivity. The day-night rhythm largely determines sleep quality. Therefore, re-aligning the timing of food intake and activity with the internal rhythm may also affect sleep. In this perspective, exercise is seen as an effective behavioural intervention to improve sleep. Since it can be challenging for people to implement exercise in their daily lives, in this thesis it was explored if an association exist between being generally more active (so not exercise per se) and sleep. Furthermore, it was investigated if there existed an association between the time-of-day at which participants were most active and sleep outcomes. Although association studies are not able to unravel cause-and-effect relationships, they do provide information if there could exist a relationship between two parameters. The study conducted in this thesis did, however, not show strong evidence of an association between the amount and timing of moderate-to-vigorous physical activity, and sleep. Thus, it does not appear that being active in general and/or being more active on a certain time-of-day is related to sleep.

To whom are the research results relevant?

The internal day-night rhythm is present in virtually all cells and tissues, and thereby affects whole-body physiology. Therefore, the research results discussed in this thesis are relevant to a wide public. First of all, the studies performed in this thesis investigated a range of mechanisms underlying the metabolic benefits of restoring the fasting-feeding cycle, but also revealed gaps in knowledge that need to be addressed in future studies. As such, academics could use the results found in this thesis to give direction to the development of future studies. Important to note is that the effects of reinforcing the internal rhythm via timed interventions could extend beyond metabolic health. Indeed, rhythmicity can be found in the whole body and disturbances of the internal rhythms do not only affect metabolism. For example, sleep deprivation and disruption of the day-night rhythms are also implicated in the development of Alzheimer's disease, which is the third leading cause of death in America and Europe. Conversely, people with Alzheimer's disease also exhibit disruptions in their internal rhythm. Thus, an interesting future research direction could be the effect of re-aligning the internal rhythm with the external rhythm via time restricted eating on the prevention or management of Alzheimer's disease.

Secondly, the studies performed in this thesis could be relevant to the field of medicine. In this perspective, it has long been known that some medication work better when taken at a specific time-of-day, termed chronotherapy. However, there are still a lot of knowledge gaps in the field of chronotherapy when it comes to metabolism. Currently, there are agents on the market that are able to partly mimic the effects of exercise and/or fasting by targeting the same metabolic pathways. Results in this thesis support the notion that altering the timing of the intake of these agents may enhance their efficacy. Thus, in this thesis is was showed that creating a more fasted state during the night resulted in beneficial metabolic outcomes, such as a higher fat oxidation and improved glucose homeostasis. Therefore, timing the intake

of exercise/fasting mimetics so that they are effective at night time may confer the maximal metabolic benefit.

Ultimately, it is not unlikely that the timing of food intake and physical activity could also be combined with medical treatment to gain the maximal metabolic benefit. Thus, in the future, medicine and lifestyle may join forces to achieve the highest treatment efficacy. Furthermore, results in this thesis may not only be relevant for medical research, but also for daily medical practice. The current modus operandi for assessing the medical status of a patient is the examination of the patient during only one medical visit. However, studies in this thesis show that fasting time as well as the time-of-day at which the measurement takes place can affect its outcome. Twenty-four-hour measurements, performed over multiple days, could give a better indication of the health status of a patient as compared to a single measurement at one time point. With the introduction of devices that are able to measure glucose every 15 minutes for multiple days, the development of devices that are able to also measure other metabolites for multiple days seems within arm's reach.

Thirdly, when looking at health from a broader perspective, the studies discussed in this thesis may be interesting for the public health sector as well. An important take home message from our results is that a long fasting period during the night positively affects metabolism, which could be implemented into a healthcare recommendation that is easy to understand. Given that fasting does not require any resources, the recommendation of prolonging the fasting period also fulfils the societal demand for sustainability.

Finally, a lot of media attention was gained for the TRE study, indicating that the results in this thesis are not only relevant for professionals working in the field of healthcare and type 2 diabetes, but that also the general population expresses an interest in these findings.

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About the author

Charlotte Andriessen was born on August the 28th 1990 in 's-Hertogenbosch, the Netherlands. From 2011-2014, Charlotte was enrolled in the BSc Health Sciences at Maastricht University. As part of her bachelor thesis, Charlotte performed a pilot study under the supervision of Prof. dr. Wouter van Marken Lichtenbelt. After successful completion of the BSc, Charlotte moved to Wageningen to study the MSc Nutrition and Health at Wageningen University and Research Centre. During her MSc, Charlotte was enrolled in the VLAG Research Master Track which is an honours programme that prepares motivated students for a career in academia. Furthermore, as part of the MSc, Charlotte completed a 4-month internship at the University of Copenhagen where she wrote a scientific manuscript under the supervision of dr. Pia Christensen and Prof. dr. Anne Raben. In 2018 Charlotte started her PhD at the department of Nutrition and Movement Sciences under supervision of prof. dr. Patrick Schrauwen, dr. Joris Hoeks, and prof. dr. Vera Schrauwen-Hinderling. Charlotte is a clinical researcher who also has a keen interest in epidemiology. Her research expertise entails human circadian biology, type 2 diabetes, metabolism, lifestyle interventions, and nutrition. Charlotte will continue her academic career at Amsterdam UMC as a post-doc, where she will be involved in an implementation study on lowering glucose-lowering medication in elderly who receive too much medication.