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Improving flexibility in substrate metabolism

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Improving flexibility in substrate metabolism:

A pharmacological and lifestyle approach

Anna Veelen

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Improving flexibility in substrate metabolism:

A pharmacological and lifestyle approach

DISSERTATION

to obtain the degree of Doctor at the Maastricht University,

on the authority of the Rector Magnificus,

Prof. dr. Pamela Habibović

in accordance with the decision of the Board of Deans,

to be defended in public

on Wednesday 25 January 2023, at 10.00 hours

by

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CHAPTER 1

General introduction and outline

Obesity and Type 2 diabetes

Overweight and obesity are health problems reaching pandemic proportions. Over the last 4 decades, the prevalence of overweight and obesity has nearly tripled. In 2016, 39% of the adults were overweight and 13% were obese (1). The major underlying cause of overweight and obesity is a change in lifestyle leading to a misbalance between energy intake and energy expenditure. Overweight and obesity are associated with several chronic metabolic diseases, including cardiovascular disease, non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (2, 3). In 2021, 537 million people worldwide were estimated to have diabetes, and it is expected that this will increase to 643 million people in 2030 and 783 million people in 2045, an increase of 46% (4). Around 90% of these numbers are accounted for by type 2 diabetes.

One of the hallmarks of type 2 diabetes is resistance to the actions of the hormone insulin, especially in skeletal muscle, liver and white adipose tissue. In the initial stage of insulin resistance pancreatic β-cells increase insulin secretion to maintain normoglycemia, however, over time the increased insulin secretion cannot overcome insulin resistance anymore and the plasma glucose levels gradually start to rise, resulting in hyperglycemia. These elevated plasma glucose levels originate from a blunted insulin-stimulated glucose uptake by predominantly the skeletal muscle, as well as a blunted insulin-mediated suppression of hepatic glucose production (5). Furthermore, insulin resistance in white adipose tissue is reflected by decreased insulin-mediated lipid storage and diminished suppression of lipolysis, thereby increasing plasma free fatty acid levels (6, 7). These circulating free fatty acids lead to storage in non-adipose tissues, such as the liver and skeletal muscle. This storage in non-adipose tissues is referred to as ectopic fat storage and exacerbates skeletal muscle and liver insulin resistance, which associates with decreased mitochondrial fat oxidative capacity (8).

Mitochondrial function and substrate oxidation

Mitochondria are cellular organelles involved in aerobic energy production within almost all cells in the body. Mitochondria oxidize substrates, mainly glucose and fatty acids, to form adenosine triphosphate (ATP). ATP contains chemical energy which can be used for a wide range of processes within the cell. Substrate availability and energy requirements are constantly changing; therefore, mitochondria are capable of switching from predominantly fat oxidation to predominantly carbohydrate oxidation, and vice versa. This switch is referred to as metabolic flexibility (9). Insulin resistance is associated with a blunted switch in substrate oxidation, or metabolic inflexibility (10). In the metabolically inflexible state, the switch from fat oxidation in the fasted state to carbohydrate oxidation in the postprandial state is blunted. This results in reduced fat oxidation and elevated carbohydrate oxidation rates during the overnight fasting state and elevated fat oxidation and reduced carbohydrate

oxidation under insulin-stimulated conditions. A normal 24-hour rhythm is reflected by having good metabolic flexibility, defined by periods of eating and periods of fasting.

It has been speculated that during a 24-hour cycle especially the nocturnal period of fasting, with high fat oxidation rates and hepatic endogenous glucose production, is important to maintain metabolic health. In the fasted state, hepatic endogenous glucose production is stimulated to maintain normoglycemia. This glucose can be produced by breaking down hepatic glycogen storages, in a process called glycogenolysis, or by novel production of glucose from non-carbon sources, referred to as gluconeogenesis. During the overnight fast, both processes contribute for approximately half of the hepatic glucose production (11). Depletion of the hepatic glycogen stores is linked to metabolic adaptations including increased fat oxidation (12). Therefore, treatment strategies to induce a more pronounced overnight fasting state may be beneficial, as these could promote hepatic glycogen depletion and consequently fat oxidative capacity of the mitochondria.

Possible treatments to promote a more pronounced fasting state

A possible treatment strategy to induce a more pronounced overnight fasting state is prolonging the overnight fasting time. By doing this, the time between the last meal of the day, and the first meal of the next day is extended, probably resulting in enhanced hepatic glycogen depletion. Indeed, time-restricted eating is very popular and results in enhanced fat oxidation (13) and insulin sensitivity (14). However, whether hepatic glycogen depletion is underlying these improvements is unknown. Another possible treatment strategy to promote a more pronounced fasting state is via sodium-glucose cotransporter 2 (SGLT2) inhibitors. SGLT2 inhibitors are a class of medication used in the treatment of type 2 diabetes. SGLT2 inhibitors promote urinary glucose excretion, thereby losing energy and creating an energy deficit, potentially promoting a more pronounced fasting state. It has been reported that in patients with type 2 diabetes, treatment with SGLT2 inhibitors results in a higher 24-hour and nocturnal fat oxidation (15-18), but also higher diurnal glucagon, free fatty acids, and β-hydroxybutyrate levels, and lower diurnal glucose and insulin levels (18, 19). Furthermore, an elevated endogenous glucose production has been observed, which could originate from increased overnight glycogen depletion. Therefore, SGLT2 inhibitors might be a good candidate to elicit a more pronounced overnight fasting state.

Thesis outline

The aim of this thesis is to investigate whether a more pronounced overnight fast can improve metabolic health. The focus lies with the effects exerted on substrate oxidation and hepatic glycogen stores.

In **chapter 2** the heterogeneous pathogenesis of type 2 diabetes and the currently available second-line medications for the treatment of type 2 diabetes are reviewed. Type 2 diabetes is a very heterogeneous disease, but the treatment strategies are only based on achieving glycemic control and preventing end-organ damage. The working mechanism and clinical available data on insulin resistance and β-cell function of each type of medication are reviewed and linked to the metabolic disturbances in type 2 diabetes with the aim to provide a first step towards personalized medicine in the treatment of type 2 diabetes.

As outlined above, SGLT2 inhibitors may be a potent pharmacological agent to induce a more pronounced fasting state. Therefore, in **chapter 3** the effects of the SGLT2 inhibitor dapagliflozin on 24-hour and nocturnal substrate metabolism, hepatic glycogen depletion, and muscle mitochondrial function were investigated. This double-blinded, placebo-controlled randomized control trial was performed in individuals with prediabetes, as the information on SGLT2 inhibition in this population is limited.

Next to the pharmacological restoration of the eating to fasting cycle, the timing of food intake can also be used to induce a longer period of fasting. In **chapter 4** a proof-of-concept study was performed to investigate whether acutely prolonging the overnight fasting time stimulates overnight hepatic glycogen depletion and whether this leads to changes in overnight and postprandial substrate metabolism.

In **chapter 5** it was examined time-restricted eating can exert beneficial effects for patients with type 2 diabetes when time-restricted eating is maintained for a longer period. Therefore, the effects of 3-weeks of time-restricted eating on substrate metabolism, overnight glycogen depletion and glucose homeostasis were investigated in patients with type 2 diabetes.

As outlined above, a disturbed eating-fasting cycle is mainly characterized by disturbed substrate oxidation during the night. We previously showed that metabolically compromised older individuals had low rates of nocturnal fat oxidation and high rates of carbohydrate oxidation compared to healthy young individuals (20, 21). However, which participant characteristics are responsible for this low nocturnal fat oxidation is still largely unknown. Therefore, in **chapter 6** it was investigated which participant characteristics can predict nocturnal RER. To this end, a combined data analysis was performed with the nocturnal data presented in chapters 3, 4, and 5 of this thesis, together with data from previously performed clinical trials within our research group.

Finally, in **chapter 7** the main findings of this thesis are revisited and placed into a broader perspective.

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CHAPTER 2

Type 2 diabetes subgroups and potential medication strategies in relation to effects on insulin resistance and beta-cell function: A step toward personalized diabetes treatment?

Anna Veelen, Edmundo Erazo Tapia, Jan Oscarsson, and Patrick Schrauwen

A.V. and E.E.T contributed equally to this work

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ABSTRACT

Background

Type 2 diabetes is a syndrome defined by hyperglycaemia that is the result of various degrees of pancreatic β-cell failure and reduced insulin sensitivity. Despite the fact that diabetes can be caused by multiple metabolic dysfunctions, the majority of patients are defined as having either type 1 or type 2 diabetes. Recently, Ahlqvist and colleagues proposed a new way to classify patients with adult-onset diabetes, taking the heterogenous metabolic phenotype of the disease into account. This new classification system could be useful for a more personalized treatment based on the underlying metabolic disruption of the disease, although so far no prospective intervention studies have generated data to support such a claim.

Scope of Review

In this review, we first provide a short overview of the phenotype and pathogenesis of type 2 diabetes, and discuss the current and new classification system. Further, we aim to review the effects of different antidiabetic medication classes on insulin sensitivity and β-cell function and discuss future treatment strategies based on the subgroups proposed by Ahlqvist *et al.*

Major Conclusions

The proposed novel subgroups of type 2 diabetes provide an interesting concept that could lead to a better understanding of the pathophysiology of the broad group of type 2 diabetes paving the way for personalized treatment choices based on understanding the root cause of the disease. We conclude that all novel subgroups of adult-onset diabetes would benefit from antidiabetic medication that take into account the main pathophysiology of the disease and thereby prevent of end-organ damage. However, we are just in the beginning of personalized treatment of type 2 diabetes and studies to investigate effects of current and novel drugs in subgroups with different metabolic phenotypes is needed in order to develop personalized treatment of the syndrome.

1. INTRODUCTION

Type 2 diabetes mellitus (T2D) is a global health problem that according to the International Diabetes Federation will affect 700 million people in 2045 (1, 2). Treatment requires a multidisciplinary approach aiming to prevent and decrease the risk of complications. Glucose-lowering medication is a key element towards the control of blood glucose levels. Increased blood glucose levels in T2D is explained by a combination of insulin resistance and reduced β-cell function. In some T2D patients' insulin resistance predominates and in other patients reduced insulin secretion is the main dysfunction. The mechanisms underlying β-cell failure and reduced insulin sensitivity are multi-faceted. Despite these multifactorial aspects of the disease, treatment options are still relatively limited and often not personalized towards the underlying causes of hyperglycaemia. Importantly, T2D is a systemic syndrome affecting almost all tissues in the body and the disease is associated with increased risk for many diseases including cardiovascular (CV) diseases, kidney disease, non-alcoholic fatty liver disease (NAFLD) as well as Alzheimer's disease and various cancers. Until now, none of the glucose-lowering medications have had any major impact on end-organ protection. However, recent studies have shown that sodium-glucose cotransporter 2 (SGLT2) inhibitors and glucagon-like peptide-1 receptor agonist (GLP-1RA) reduce the risk for CV disease showing end-organ protection beyond glucose lowering. In this review, we aim to provide a short overview of the pathogenesis and classification of T2D, effects of medication classes on insulin sensitivity and β-cell function and aim to provide future treatment perspectives.

2. PATHOGENESIS OF DIABETES

Type 2 diabetes is a disease that includes multiple metabolic dysfunctions characterized by hyperglycaemia that is the result of various degrees of pancreatic β-cell failure and reduced insulin sensitivity. Risk factors for development of T2D include obesity, sedentary lifestyle and associated insulin resistance. However, most obese and insulin resistant individuals never develop T2D that is explained by strong genetic components associated with T2D. As presented by DeFronzo in 1988 (3), the development from impaired glucose tolerance (IGT) towards T2D is mainly the result of a decreased β-cell function, and not due to an altered insulin mediated glucose uptake, whereas insulin resistance is often already present before hyperglycaemia and a rise in HbA1c occurs. However, it should be pointed out that treatment of insulin resistance would reduce the β-cell burden and improve hyperglycaemia. The risk for developing T2D is strongly inherited, and many genetic associations have been described, although they today explain just a fraction of the genetic association (4). Most of the genetic associations have been ascribed to β-cell function and very few has been linked to insulin resistance (4), although this may be partly also due to the fact that no good measures of insulin sensitivity are available in large cohorts.

In T2D, β-cell failure has been shown to be associated with a 24 - 65% loss of β-cell mass, but also with a 50 - 97% loss of insulin secretory capacity of the β-cells (5). Pancreatic β-cells initially overcome insulin resistance of peripheral tissues by producing more insulin, leading to supraphysiological insulin concentrations. Over time, β-cell failure occurs, leading to elevated postprandial and fasting glucose levels in spite of continued hyperinsulinemia. Mechanisms that have been associated with β-cell failure are, among others, insulin resistance, glucotoxicity, lipotoxicity, β-cell senescence (6, 7), dedifferentiation (8) and/or apoptosis (9, 10). First-degree relatives to T2D have a dysregulated insulin secretion, with less regular pulsatility of insulin secretion (11). This change in insulin pulsatility may result in down-regulation of insulin action and indicates an interaction between dysregulated β-cell function and worsening of insulin resistance (12). Therefore, it is not fully clear if insulin resistance proceeds β-cell failure in all individuals who develop T2D.

The other major hallmark in the development of T2D is the gradual development of whole-body and peripheral insulin resistance. As skeletal muscle, the largest organ of the body, is responsible for approximately ~85% of postprandial glucose uptake, skeletal muscle insulin resistance contributes to the development of hyperglycaemia (13). In skeletal muscle, insulin resistance is characterized by a reduced intracellular insulin-stimulated glucose uptake and handling, among others due to a reduced insulin-induced GLUT4 translocation to the cell membrane and subsequent glycogen synthesis (figure 1) (14).

Besides skeletal muscle, liver insulin resistance results in elevated basal endogenous glucose production (EGP) and reduced insulin-suppression of EGP, thereby further contributing to higher plasma glucose concentrations (figure 1) (9). Adipose tissue insulin resistance contributes to hyperglycemia by reduced glucose uptake, although adipose tissue glucose uptake is generally considered relatively small in humans (15). However, adipose tissue insulin resistance also leads to reduced inhibition of lipolysis by insulin, which results in elevated free fatty acids (FFA) level in the blood (figure 1) (16, 17). High circulatory FFA can contribute to skeletal muscle insulin resistance (see below). Furthermore, higher rates of lipolysis also give rise to higher levels of glycerol, which are considered an important source for gluconeogenesis and EGP (18). Please see figure 1 for an illustration of common changes in postprandial insulin action in type 2 diabetes.

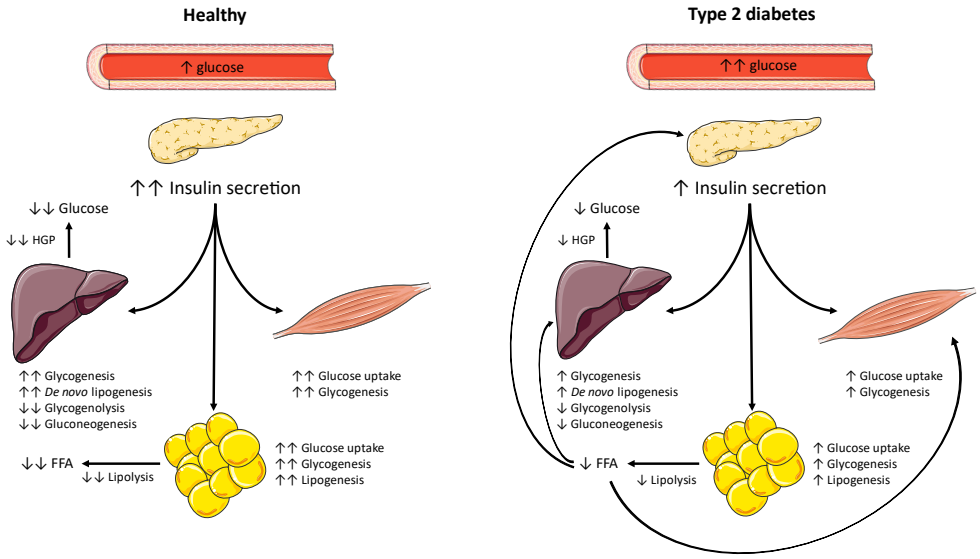

Figure 1: Action of insulin in the postprandial state in a healthy and type 2 diabetes conditions. Increasing blood glucose will lead to the secretion of insulin. Insulin stimulates glucose uptake in skeletal muscle and white adipose tissue and suppresses lipolysis in white adipose tissue leading to a reduction in circulatory free fatty acid (FFA) levels. In liver, insulin and a reduced adipose lipolysis suppresses hepatic glucose production (HGP), via a combination of reductions in gluconeogenesis and glycogenolysis and stimulation of glycogen storage. The combined action of glucose uptake and reduction in HGP contributes to plasma glucose control. In type 2 diabetes, glucose-induced insulin secretion is not sufficient due to reduced β-cell function and insulin-stimulated glucose uptake in muscle and white adipose tissue (WAT), as well as insulin-stimulated suppression of HGP, is blunted. In addition, insulin resistance in WAT leads to blunted suppression of lipolysis by insulin, leading to higher FFA levels, which subsequently negatively affect skeletal muscle and HGP. FFA, free fatty acids; HGP, hepatic glucose production.

3. UNDERLYING CAUSES OF THE DEVELOPMENT OF β-CELL FAILURE, INSULIN RESISTANCE AND T2D

T2D is strongly associated with obesity, and ~ 90% of all T2D patients are overweight or obese. Expansion of fat mass serves to ensure the storage of excess nutrients/energy; however, when adipose tissues expandability becomes limiting or dysfunctional (19) circulating FFA and elevated uptake of FFA in liver and skeletal muscle can occur, where they can compete with glucose for substrate oxidation and according to the Randle cycle can contribute to insulin resistance (20). Furthermore, FFA can also accumulate in non-adipose tissues, and such ectopic fat accumulation has been shown to be a crucial factor in the development of insulin resistance in the liver and skeletal muscle, mainly due to the interference of diacylglycerol and ceramides among others, with the insulin-signalling pathway (21-23). Increased uptake of FFA is also associated with oxidative stress, inflammation, and cell death. Lipotoxicity can

occur in a range of tissues such as skeletal muscle, heart, arteries, pancreas and liver, creating different phenotypes/end-organ damage among patients, depending on which organs are most affected. In muscle, fat accumulation interferes with insulin-stimulated GLUT4 translocation and in the liver, non-alcoholic fatty liver (NAFL), is associated with hepatic insulin resistance and enhanced production of VLDL-TG that contributes to development of atherogenic/diabetic dyslipidemia (24, 25). As mentioned before, the development of hepatic insulin resistance could also be due to deficient pulsatile insulin delivery into the hepatic portal vein and finally to the hepatocytes (12). This hypothesis suggests that dysregulated insulin delivery, which is present in T2D, could lead to dysregulation of hepatic lipid metabolism or selective insulin resistance through FoxO1 contributing to the accumulation of lipids (26). Selective insulin resistance refers to the pathological state in which insulin does not decrease hepatic glucose production, but insulin stimulation of de novo lipogenesis via activation of SREBP-1c is unaffected and further increased due to the associated hyperinsulinemia, leading to hepatic fat accumulation (27). In the pancreas, β-cell exposure to chronic high levels of FFA lead to endoplasmic reticulum (ER) stress and mitochondrial dysfunction, which can result in cell damage and eventually impaired insulin secretion (28).

Chronic hyperglycaemia has also been shown to exert toxic effects on β-cells and other tissues, a phenomenon termed glucotoxicity. Glucotoxicity contributes to β-cell failure, but also to reduced insulin sensitivity in the liver via different processes, such as ER stress, mitochondrial dysfunction, oxidative stress, and inflammation (10, 29). Besides chronic hyperglycaemia, glycogen storage in β-cells has been shown to be associated with apoptosis (30). Whether glucotoxicity also has effects on skeletal muscle insulin sensitivity is still under debate and not fully understood (14).

Apart from obesity, age is another determinant of the development of T2D and T2D has also long been considered a disease associated with accelerated ageing. Wijsman *et al.* (31) showed that familial longevity was characterized by better insulin sensitivity compared to a group with same age, sex and body composition. With age often a decrease in physical activity and muscle mass is observed, factors that directly contribute to the development of skeletal muscle insulin resistance. In addition, ageing is often associated with an increase in fat mass, which could contribute to the development of lipotoxicity and insulin resistance. Cellular stress responses can lead to a state of cellular senescence characterised by cell-cycle arrest, resistance to apoptosis and a senescence-associated secretory phenotype (SASP), which negatively influence organ functions. It has been shown that insulin resistance accelerated β-cell senescence in human islets (Aguayo-Mazzucato). Moreover, in a mouse model of type 1 diabetes, it was shown that elimination of senescent cells halted immune mediated β-cell destruction and prevented diabetes (32). Thus, both improved insulin sensitivity and enhanced apoptosis of senescent islet cells could improve β-cell function.

The Baltimore Longitudinal Study of Aging showed that insulin secretion decreases with age independent of BMI and adipose tissue distribution (33). The latter could explain why the prevalence of T2D is associated with increasing age in the population.

As stated above most insulin resistant people do not develop T2D and genetic components could explain why some insulin resistant people develop T2D. Genome-wide association analysis have identified single-nucleotide polymorphisms (SNP´s) that are associated with the function of the β-cell. Some of these genetic variants are located over 40 loci and can increase the risk for T2D. Even though more than 400 gene variants have been associated with the presence of T2D, the currently identified variants account for only 10% of the genetic influence for the risk of developing T2D (34). In contrast, maturity-onset diabetes of the young is monogenic diabetes and accounts for 2 to 5 % of all diabetes patients (35).

4. CLASSIFICATION OF DIABETES

In 1979, an international workgroup came up with a new classification system, which included Type 1 Diabetes Mellitus (T1D), T2D and gestational diabetes (36). Furthermore, they also added the IGT group; people who did not meet the criteria for diabetes mellitus but do have elevated fasting and 2-hour glucose values. In 1997, the classification system was reviewed again and the IGT group was split in two; impaired fasting glucose (IFG) and IGT (37).

Today, more than 40 years after the classification system was first suggested, the knowledge about the complexity of diabetes pathophysiology has increased. However, there are still only two major classifications: T1D and T2D. Nowadays, with the call for more a personalised medication strategy, a more refined classification system would be helpful to develop novel drugs correcting the root-cause of the syndrome, as well as prescribing the best current medication to prevent progression of the disease and end-organ damage.

In 2018 Ahlqvist *et al.* (38) suggested a new classification system of adult-onset diabetes, which, at least partly, takes the heterogeneous phenotype of T2D into account. In their subgroup classification, adult-onset diabetes is classified into five subgroups or clusters by using 6 fairly common measures that could be obtained in clinical care: BMI, age at diagnosis, HbA1c, glutamic acid decarboxylase antibodies (GADA) and homeostasis model assessment 2 (HOMA2) to estimate β-cell function (HOMA2-B) and insulin resistance (HOMA2-IR), based on fasting glucose and C-peptide concentrations. Data-driven non-supervised cluster analysis was done using large Swedish and Finnish cohorts which included all new incidents of adult-onset diabetes. This data-driven cluster analysis concluded 5 novel subgroups for newly diagnosed adult-onset diabetes based on the above-mentioned variables: severe autoimmune diabetes (SAID), severe insulin-deficient diabetes (SIDD), severe insulin-

resistant diabetes (SIRD), mild obesity-related diabetes (MOD) and mild age-related diabetes (MARD) (figure 2).

This data-driven cluster analysis concluded 5 novel subgroups for newly diagnosed adult-onset diabetes based on the above-mentioned variables: severe autoimmune diabetes (SAID), severe insulin-deficient diabetes (SIDD), severe insulin-resistant diabetes (SIRD), mild obesity-related diabetes (MOD) and mild age-related diabetes (MARD) (figure 2).

SAID and SIDD were both characterized by an earlier onset of diabetes, a relatively low BMI, poor metabolic control (high HbA1c) and insulin deficiency (determined by a low HOMA2-B index). The difference between SAID and SIDD is the presence of glutamic acid decarboxylase antibodies in SAID but not in SIDD. Severe autoimmune diabetes (SAID) overlap with both T1D and latent autoimmune diabetes in adults (LADA). The latter share genetic features with T1D, but in a clinical setting they often share characteristics of T2D patients and therefore often diagnosed as T2D. Applying the same clustering system in an independent German cohort revealed patients allocated to the SIDD group also show signs of autoimmunity (39).

SIRD is characterized by a higher BMI (overweight to obesity) and marked insulin resistance (determined by a high HOMA2-IR index). In SIRD, the β-cell function is less impaired than in SAID and SIDD (high HOMA2-B index) and HbA1c levels are lower. Both SIDD and SIRD were previously diagnosed as T2D, and represent very different forms of severe T2D.

Mild obesity-related diabetes (MOD) and mild age-related diabetes (MARD) both are characterized by relatively mild insulin resistance (HOMA2-IR lower than SIRD) and mild insulin deficiency (HOMA2-B higher than SAID and SIDD, but lower than SIRD). The difference between MOD and MARD is based on the age of diagnosis and on BMI; MOD is characterized by a high BMI (obesity), while MARD has a higher age at diagnosis. Thus, SAID constitutes patients that are today diagnosed with T1D or LADA, while patients belonging to the other four clusters are today diagnosed with T2D.

The disease progression and risk of end-organ damage seem to differ by subgroups. SAID and SIDD have a higher HbA1c at baseline and during follow-up compared to the rest of the subgroups and also associated with an increased risk of ketoacidosis (38, 39). SIRD is associated with a high prevalence of NAFLD and fibrosis at diagnosis (38, 39) as well as diabetic kidney disease and end stage renal disease (38), but when corrected for baseline kidney function, there was no difference between the different subgroups (40). In other words, patients with SIRD develop end-organ damage before they are diagnosed with diabetes. In contrast, neuropathy and retinopathy are more associated with the SIDD group (38, 39). The subgroups also differ by the initial treatment prescribed in the cohort at the time of diagnosis. In the SAID group 42 - 67% were on insulin treatment and 29 - 44% of SIDD patients were on insulin treatment (38, 39).

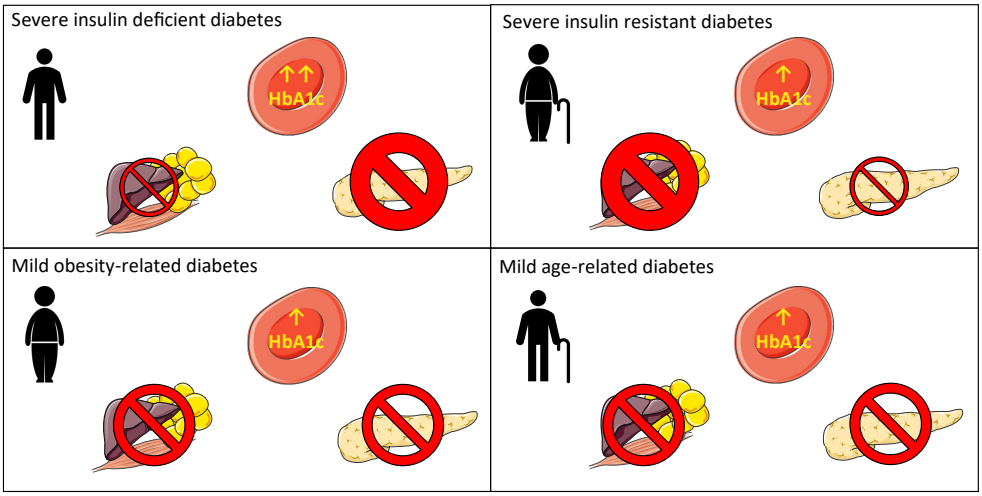

Figure 2: Visual representation of the characteristics of the subgroups as suggested by Ahlqvist *et al.* (38). Severe insulin deficient diabetes (SIDD) is characterized by a relatively low age and BMI, a high HbA1c, less marked insulin resistance but severe β-cell insulin deficiency. Severe insulin resistant diabetes (SIRD) is characterized by relatively high age and BMI, a relatively low HbA1c, severe insulin resistance but no insulin deficiency. Mild obesity-related diabetes (MOD) is characterized by a relatively low age at diagnosis, a high BMI, relatively low HbA1c and mild insulin resistance and insulin deficiency. Mild age-related diabetes (MARD) is characterized by a high age at diagnosis, a relatively low BMI and mild insulin resistance and insulin deficiency. A more severe insulin resistance/deficiency is indicated with a bigger stop sign.

5. CURRENT TREATMENT STRATEGIES

Despite the fact that T2D is an heterogenous syndrome as indicated by large inter-individual differences with respect to insulin resistance, β-cell function and autoimmunity, the current treatment strategies focus mainly on lowering glucose and HbA1c and to prevent end organ damage. As atherosclerotic cardiovascular disease (ASCVD) is still the leading cause of morbidity and mortality among patients with T2D, guidelines clearly indicate to what extent the different medications have proven to reduce the risk for CV events. Other end-organ diseases associated with T2D such as chronic kidney disease (CKD), NAFLD, neuropathy and retinopathy are also relevant complications to take into account when choosing the right therapy to the patients with T2D. However, today it is not fully understood how to predict disease progression or risks for end-organ damage in individuals with T2D. It would be better for the patients and more cost-effective if there were better ways to predict risks in order to more aggressively treat patients with larger risk as compared to patients with low risk.

Following initial recommendations regarding lifestyle modifications, weight loss and an increase in physical activity, and patient conditions are the reference elements when choosing an anti-hyperglycaemic medication. Guidelines usually consider elements such as

the patient risk of having a cardiovascular event, weight, and risk of hypoglycaemia when selecting anti-diabetic drugs.

Other elements that drive the decision are the costs of medication and proven efficacy. Therefore, until the last combined guidelines of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) of 2020, metformin continues to be the first-line therapy given the medication's unique profile when looking at cost-effectiveness and tolerability (41-43). The mechanism of action of metformin on glucose control is not fully elucidated and both the liver and the intestine has been suggested as the main target tissues; the working mechanism of metformin has been extensively reviewed previously, for further reading see (44). Nevertheless, a large proportion of patients will not be able to achieve treatment targets only by taking metformin only and eventually will require a second-line therapy addition.

The choice of second-line therapy will depend mainly on the patient having established ASCVD, CKD or heart failure (HF). If these conditions are not in place, the decision is rather based on the risk of adverse events such as hypoglycaemia, weight gain, cost and patient preferences. However, there is not much evidence to guide the choice of a second or even third agent to control glucose homeostasis.

The novel diabetes classification system suggested by Ahlqvist *et al.* (38) shows the heterogeneity of diabetes and focuses on several different factors, including insulin resistance and β-cell dysfunction. This new classification can help in developing a new, more personalized treatment approach by exploring the relationship between anti-hyperglycaemic medications and their effects on the mechanistic causes of T2D. With this classification may also follow that the patients at higher risk get a more aggressive treatment already at diagnosis to prevent the end-organ damage associated with this subtype of diabetes. At this moment, there are 5 different classes of second-line anti-hyperglycaemic medications recommended by the ADA and EASD: dipeptidyl peptidase 4 (DPP-4) inhibitors, GLP-1RA, SGLT2 inhibitors, sulfonylureas and thiazolidinediones. These medications all have been successful on the market due to their ability to improve glucose homeostasis and reduce HbA1c, but all have a unique – sometimes incompletely discovered - mechanism of action and improve glucose homeostasis in different ways. This provides opportunities for a more personalized medication treatment strategy. Therefore, below we aim to provide an overview of the proposed working mechanisms of currently prescribed second-line anti-hyperglycaemic medication and review the available clinical data on the effects of these medications on β-cell function and insulin sensitivity.

We will suggest potential treatment strategies for the novel subgroups SIDD, SIRD, MOD and MARD, today comprising the large group of T2D, as these groups may benefit from different treatment medications. Importantly, there is very little data to make the right choices for

patients depending their metabolic phenotype. Therefore, the below suggestions are hypothesise generating, and should not be regarded as recommendations.

The treatment strategies for the novel subgroup SAID will not be discussed as this group includes T1D and LADA and entails a heterogeneous group which today requires insulin therapy. Sulfonylureas will not be discussed further in this review because the effects on β-cell function and insulin sensitivity are well established. Treatment with sulfonylureas has no effect on insulin sensitivity, but it will improve β-cell function initially. After 1-2 years of therapy, HbA1c levels are raising again, indicating an worsening of the β-cell function (45). On the other hand, it should be noted that sulfonylurea has been shown to have most beneficial effects on HbA1c in patients with MARD (40), showing that sulfonylureas has a place also for long term treatment of this subgroup of T2D. Also, insulin therapy will not be discussed as we consider it is extensively reviewed and guidelines state when and under which considerations, insulin has an advantage over other second-line agents (41). Medications that improve glucose levels, including insulin therapy, allow the rest of the β-cell by compensating for the insulin demand to correct hyperglycaemia. The β-cell rest concept is beyond the context of this review but it is relevant to mention that currently there is no clinical evidence that any medication change the disease progression in terms of improving β-cell function beyond the acute effects (46, 47). However, it is worth mentioning that insulin use in T2D patients has some disadvantages such as the risk for an increase in weight, which may cause insulin resistance, and insulin therapy of patients with T2D may increase the risk of cardiovascular complications (48).

Below, we will focus on the potential second-line therapies for T2D and mainly include human trials using hyperinsulinemic clamps or mixed meal tests whenever this is possible, as these techniques are regarded as the gold-standard methods to assess β-cell function and insulin sensitivity.

5.1. Sodium-glucose cotransporter 2 (SGLT2) inhibitors

Inhibitors of SGLT2 are a novel type of glucose-lowering medications acting on SGLT2, which are expressed in the first segment of the proximal tubule in the kidneys. SGLT2 is responsible for about 90% of the glucose reabsorption of the kidneys. Inhibition of SGLT2 results in a urinary excretion of 60 – 80 grams glucose per day, the exact amount depending on plasma glucose concentrations and glomerular filtration rate, leading to a reduction in HbA1c of 0.6 – 0.9% and fasting glucose of 1.1 – 1.9 mmol/L, compared to placebo (49).

The mechanism via which SGLT2 inhibitors lower glucose levels is simple and direct, via increased loss of glucose via the urine; a mechanism that is independent of insulin action (49-51). The glucose and energy loss will trigger adaptive responses that may contribute to the beneficial effect of this group of drugs. Use of SGLT2 inhibitors is associated with a reduced

body weight (49), reduced blood pressure, and positive outcomes on CV death, HF and progression of CKD (52-54).

5.1.1. SGLT2 inhibitors and β-cell function

The urinary glucose loss may improve β-cell function via reduced glucotoxicity and/or via a reduction in excessive insulin secretion due to lower glucose concentrations (55). Although SGLT2 inhibitors do not directly target the β-cell, the effects of SGLT2 inhibition on β-cell function has been investigated in several human intervention studies. Both Al Jobori *et al.* and Merovci *et al.* reported a 2-fold increase in β-cell function, measured as improvement of insulin secretion/insulin resistance index (also called deposition index; the change in C-peptide concentration divided by the change in glucose concentration (ΔC-peptide/Δglucose) divided by the insulin resistance), after 2 weeks of SGLT2 inhibitors treatment in patients with T2D (56-58). In line, Forst *et al.* reported in two independent studies an improved β-cell function, assessed as improvements in area under the curve for insulin, C-peptide and C-peptide/pro-insulin ratio during an hyperglycaemic clamp, after 30 days of treatment with SGLT2 inhibitors in patients with T2D co-treated with metformin (59, 60).

Several studies showed that treatment with SGLT2 inhibitors improves β-cell glucose sensitivity. Thus, Ferrannini *et al.* (61) reported a 25% increase in β-cell glucose sensitivity after only 48h of SGLT2 treatment in patients with T2D who were treatment naive or treated with metformin. After 14 days of treatment, the improvements in β-cell glucose sensitivity were sustained. Also three other studies in patients who were treatment naive or given diet advice, metformin, sulfonylureas or a combination of metformin and sulfonylureas, reported that β-cell glucose sensitivity increased after both 48 hours and 14 days of SGLT2 treatment (56, 58, 62).

As described earlier, the progression of diabetes is mainly because of the decrease in β-cell function. This means that the long-term effects of improvements in β-cell function can be monitored as no progression in deterioration of HbA1c levels. The effect of SGLT2 inhibitors on HbA1c has been established in a meta-analysis including 38 studies with a duration of ≥ 24 weeks conducted by Zaccardi *et al.* (49). On average, they reported a HbA1c reduction of 0.6 - 0.9%. When focused on the studies with a long-term duration (≥ 104 weeks) measuring HbA1c, SGLT2 inhibition produced a sustained reduction of 0.30 - 1.22% (63-67).

5.1.2. SGLT2 inhibitors and insulin sensitivity

SGLT2 inhibition could lead to improved insulin sensitity via reduction in plasma glucose and reduced body weight. Weight loss is generally associated with an improvement in insulin sensitivity and a weight loss of 1.5 - 2 kg has been reported in patients on SGTL2 inhibitor

therapy (49, 68). As discussed below, a loss of glucose via the urine may lead to a compensatory stimulation of lipid oxidation in humans, which could have impact on the distribution of excessive fat mass and a reduction in ectopic fat stores, which are strongly related to the development of insulin resistance (55).

Several studies have investigated the effect of SGLT2 inhibition on peripheral insulin sensitivity (61, 69, 70). Ferrannini *et al.* (61) reported a decrease in total glucose disposal corrected for urinary glucose excretion after acute SGLT2 inhibitor administration, which was sustained after 14 days of treatment in patients with T2D, who were either treatment naive or co-treated with metformin. However, in spite of the reduced glucose disposal, predominately caused by a decrease in non-oxidative glucose disposal, peripheral insulin sensitivity, estimated by the ratio of the glucose metabolic clearance rate to the mean plasma concentration during a mixed meal test, was significantly increased after acute administration but the increase did not reach statistical significance after 14 days of treatment. Merovci *et al.* (69) found similar results using hyperinsulinemic euglycemic clamp to assess insulin sensitivity. Fourteen days of SGLT2 inhibitor administration increased insulin-stimulated whole body glucose disposal corrected for urinary glucose loss from 4.3 ± 0.4 to 5.0 ± 0.4 mg/kg/min, which was a significant increase compared to baseline and placebo (4.0 ± 0.5 to 4.3 ± 0.6 mg/kg/min) in patients with T2D treated with either metformin or a combination of metformin and sulfonylureas. Similarly, upon 12 weeks of SGLT2 inhibitor treatment, peripheral insulin sensitivity, measured during a hyperinsulinemic euglycemic clamp, improved compared to placebo in patients with T2D co-treated with metformin or a combination of metformin and an insulin secretagogue (70).

Similar results were later found in other studies. Thus, in patients with T2D, co-treated with either metformin, sulfonylureas, DPP4-inhibitors or a combination of metformin and sulfonylureas, peripheral insulin sensitivity was improved by approximately 16 - 36% compared to baseline and placebo after SGLT2 inhibitor administration (57, 58, 71, 72). In contrast, Latva-Rasku *et al.* (73) did not find an improvement after 8 weeks of SGLT2 inhibition on insulin sensitivity (measured as whole-body insulin-stimulated M-value) or skeletal muscle glucose uptake in patients with T2D co-treated with metformin or metformin in combination with DPP-4 inhibitors. The authors indicated that the severe insulin resistance among the participants could explain why a relatively lower insulin infusion rate (40mU/m2/min) did not detect a change in M-value. Although liver fat content decreased significantly (proton density fat fraction; -3.7%), this reduction in hepatic fat did not lead to an improvement of hepatic insulin sensitivity (measured as suppression of EGP), or an enhanced glucose uptake in the liver.

Instead, several studies reported an increase in EGP upon SGLT2 inhibitor administration (61, 69-71, 74). The hepatic and possibly renal production of glucose compensates for approximately half of the glucose lost in urine in T2D patients, thereby blunting the lowering

of the plasma glucose concentrations (69). The exact mechanism leading to a compensatory increase in EGP is still unclear. It has been suggested that a decreased insulin:glucagon ratio or autonomic nervous system (ANS) mediated mechanisms could be involved. Recently, Alatrach *et al.* (75) demonstrated that insulin and glucagon concentrations under glucose clamp condition (prevention of a fall in glucose levels) did not differ between subjects receiving SGLT2 inhibitors or placebo, but SGLT2 inhibition caused an increase in EGP in contrast to placebo. This argues against an important role for the insulin:glucagon ratio in mediating the increase in EGP upon SGLT2 inhibition. Furthermore, Solis-Herrera *et al.* and Daniele *et al.* (76, 77) hypothesized that renal ANS afferents are important for the increased EGP following SGLT2 inhibition. They therefore investigated the effect of SGLT2 inhibition on EGP in kidney transplanted patients with either both residual native kidneys in place or with a bilateral nephrectomy. In both patient groups an increase in EGP upon SGLT2 inhibitor administration occurred. While the increase in EGP in patients with their native kidneys in place was comparable with other studies, the increase in EGP was blunted in the patients with a bilateral nephrectomy. This finding indicates a role for the kidneys and/or ANS in the increase in EGP, however, the mechanism leading to the increase in EGP following SGLT2 inhibition remains unclear.

SGLT2 inhibition has been reported to result in changed substrate oxidation, which may have favourable effects on insulin sensitivity. Thus, a decrease in glucose oxidation and increase in lipid oxidation and ketone production have been reported (71, 78), which could contribute to the improvements in β-cell function and insulin sensitivity by reducing ectopic fat and ameliorating lipotoxicity. On the other hand, the increased fatty acid oxidation is associated with increased adipose tissue lipolysis and increased flux of fatty acids that would reduce glucose uptake in skeletal muscle, thereby reducing skeletal muscle insulin mediated glucose uptake. However, there is limited knowledge about changes in intracellular potentially harmful lipids, and ectopic fat has to the best of our knowledge been shown to be reduced in the liver (73, 79, 80), visceral fat (81) and epicardial fat (82) following treatment with an SGLT2 inhibitor.

In conclusion, administration of SGLT2 inhibitors result in a modest, but significant increase in β-cell function and β-cell glucose sensitivity. Long-term studies indicate sustained glucose lowering after at least 2 years of treatment. To the best of our knowledge, no wash-out studies have been done to investigate if the improved β-cell function is sustained after stopping treatment. With regard to insulin sensitivity, several research groups reported an improved insulin sensitivity, but the improvements are small. It is suggested that the beneficial effects of SGLT2 treatment are mainly caused by a decreased glucotoxicity. However, the clinical trials investigating β-cell function and insulin sensitivity over a longer period of time are limited. It could well be that treatment for longer periods than 3-4 months may show a different result. For example, data suggest that after 3-4 months, the energy losses are compensated by increased food intake that would explain that body weight do not

reduce further after this period of time (83, 84). The available data on β-cell function and insulin sensitivity, and the fact that SGLT2 inhibitors works independent of insulin, suggest that SGLT2 inhibitor therapy could be beneficial in all four proposed novel subgroups of T2D. Meanwhile, the first study to investigate the efficacy of SGLT2 inhibition and a GLP-1 receptor agonist in patients with SIDD and SIRD has started to recruit (ClinicalTrials.gov Identifier: NCT04451837).

5.2. Glucagon-like peptide-1 (GLP-1) receptor agonists

GLP-1 is a hormone produced by the L-cells of the intestine in response to food ingestion, especially to meals with a high content of fat and carbohydrates. GLP-1 administration improves glucose levels through different mechanisms including glucose-dependent insulin secretion, reduction of food intake, reduced body weight and decreased levels of glucagon. Glucagon-like peptide-1 receptor agonists (GLP-1RA) reduces HbA1c in a range from 0.5 - 1.5% (85, 86).

5.2.1. GLP-1RA and β-cell function

One of the expected working mechanisms of GLP-1RA is via a direct effect on the β-cell. Indeed, β-cells express GLP-1 receptors. The GLP-1 receptors are G protein-coupled and upon activation result in increased cAMP and PKA activity, thereby promoting insulin release from the β-cell (87). The LIBRA trial assessed β-cell function in patients with recent T2D diagnosis treated with insulin for 4 weeks before randomization with either a long-acting GLP-1RA or placebo for 48 weeks and found improved β-cell function measured by insulin secretion-sensitivity index-2 in the active group (88). Another randomised controlled trial in patients with T2D compared the effect of a short-acting GLP-1RA versus placebo for three years and observed improvement in β-cell function measured by the Mari model, a method that assess β-cell function from values obtained during an OGTT (89).

Anholm *et al.* found that 12 weeks of metformin plus GLP-1RA lead to a significant increase in β-cell function - as assessed by the disposition index - compared to the metformin or placebo group in a randomized, double-blind crossover trial (90). Another randomized controlled trial investigated the effect of GLP-1RA plus metformin vs metformin plus lifestyle interventions on β-cell function in patients with recent T2D diagnosis and found that liraglutide improved β-cell function, expressed as the β-cell secretion during an OGTT, compared to the control group within a 15 month period (91). By now, the positive effects of short and long-acting GLP-1RA on β-cell function have been demonstrated in several randomized clinical trials.

In animal models of diabetes it has been shown that GLP-1RA treatment improves the function of β-cell mainly through proliferation and differentiation. (92). However, whether

GLP-1RA increases functional β-cell mass in humans is so far unknown. The results of wash-out studies (88, 89) show no lasting effect on β-cell function and therefore indicates that there is no effect on functional β-cell mass, instead the effects on β-cell function seem to be acute.

5.2.2. GLP-1RA and insulin sensitivity

Gastaldelli *et al.* (93) investigated the acute effect of a short-acting GLP-1RA on hepatic and adipose tissue insulin sensitivity, measured as glucose and glycerol tracer kinetics after a ^{13}C-enriched glucose load. The study was conducted in patients with T2D and in subjects with IGT. They found that acute treatment with GLP-1RA improved hepatic and adipose tissue insulin sensitivity when compared to placebo. Prolonged effects of GLP-1RA on insulin sensitivity was investigated by Zander *et al.* (94). They investigated the effect of continuous subcutaneous infusion of GLP-1RA versus saline infusion, using a portable pump, for 6 weeks in patients with T2D and found that insulin sensitivity measured by a hyperinsulinemic-euglycemic clamp was increased by 77%. However, this effect on insulin sensitivity could have been overestimated as the study was neither randomised nor blinded. The improvement in insulin sensitivity was accompanied by a decrease in fasting plasma glucose and FFA levels that could have contributed to the effect. Anholm *et al.* (95) investigated the effect of a GLP1-RA plus metformin versus metformin plus placebo on insulin sensitivity in obese and overweight patients with newly diagnosed T2D and coronary artery disease. Insulin sensitivity was measured by the ISI-composite, a measure of whole-body insulin sensitivity obtained from a formula that combines values derived from an OGTT and the values from fasting plasma glucose and insulin (96). GLP1-RA plus metformin increased β-cell function, measured by the disposition index, by 40% compared to metformin plus placebo, but insulin sensitivity was not significantly different between the groups (95).

Armstrong *et al.* (97) evaluated the effect of GLP-1RA on hepatic insulin sensitivity, measured as suppression of EGP, after 12 weeks of GLP-1RA treatment versus placebo in subjects with non-alcoholic steatohepatitis (NASH). Before and after treatment a hyperinsulinemic euglycemic clamp was performed, and it was found that GLP-1RA reduced EGP compared to placebo (-9.3 vs - 2.5%). GLP-1RA also significantly reduced body weight in the intervention group compared to placebo. Dutour *et al.* (98) evaluated the effect of GLP-1RA on hepatic fat content measured by magnetic resonance spectroscopy (MRS) in obese patients with T2D. After 26 weeks of treatment they found a significant reduction in hepatic fat content in the intervention group versus placebo (-23.8 % vs +12.5%). This reduction in liver fat was highly correlated to body weight loss.

Indeed, an effect of GLP-1RA on body weight may be the explanation for the beneficial effects on hepatic and peripheral insulin sensitivity that has been observed. A meta-analysis that

included 25 trials comparing GLP-1RA against placebo, insulin or other glucose-lowering medication, found that GLP-1RA leads to a significant reduction in body weight (99). The results showed a mean difference of -2.9 kg body weight loss in the intervention group compared to the control group. Davies *et al.* (100) also reported the long term effect on body weight after 56 weeks of treatment compared to placebo in overweight and obese subjects with T2D, and reported significantly larger weight loss in the intervention group compared to placebo. Other potential explanations to the effect on insulin sensitivity could be the association that has been found in animal models between GLP-1RA treatment and decrease in inflammation (101). Lynch *et al.* (102) investigated the association between GLP-1RA therapy and invariant natural killer T (iNKT) cells in human and mice adipose tissue, and observed that GLP-1RA activates iNKT cells. Interestingly, iNKT cells activation can lead to weight loss. Therefore, GLP-1RA may partly reduce body weight and improve insulin sensitivity by acting on the immune system.

In conclusion, GLP-1RA improves β-cell function during treatment, but the effect does not stay after discontinuation of the treatment (103). GLP1-RA treatment effect on glucose control seems to be mainly based on the ability to increase insulin secretion, with contribution of improved insulin sensitivity via weight loss and immune modulatory effects. However, there is limited information on changes in insulin sensitivity after GLP-1RA administration.

Current guidelines establish GLP-1RA as a second line therapy in obese patients and also in patients with diagnosis of CV disease. We suggest that GLP-1RA therapy could also be a preferred treatment option for all obese the subgroups described by Ahlqvist, including SIRD and MOD, as well as SIDD. Considering the initial nausea, GLP-1RA may be a less attractive treatment for the MARD group, also considering age of onset and less risk for developing diabetes associated end-organ damage.

5.3. Dipeptidyl peptidase 4 (DPP-4) inhibitors

DPP-4 inhibitors are a class of glucose lowering medications that inhibit the enzyme DPP-4. This enzyme is expressed on the surface of many cells such as adipocyte, kidney, liver and small intestine and it decreases the activity of peptides, such as GLP-1 and glucose-dependent insulinotropic polypeptide (GIP). DPP-4 inhibitors properties are characterized by competitive inhibition and high affinity to DPP-4. DPP-4 inhibitors reduce HbA1c in a range from 0.5 - 1% (104).

5.3.1. DPP-4 inhibitors and β-cell function

DPP-4 inhibitors' effect on glucose metabolism is thought to be mainly by increasing the availability of incretins such as GLP-1 and GIP, which are responsible for increasing insulin

secretion and decreasing glucagon secretion after a meal (105). The effect on β-cell function has been established in several clinical studies. A meta-analysis of 23 randomized placebo-controlled studies associated DPP-4 inhibitor treatment with a significant improvement in HOMA-B compared to placebo (106). When DPP-4 inhibitors were used as add-on therapy, also a significant improvement in HOMA-B was found. HOMA-B is mainly a measure of insulin secretion, and there are only a few studies that measure the effect of DPP-4 inhibitors on β-cell function using gold standard methods.

In animal models of obesity, treatment with DPP-4 inhibitors for 11 months was associated with better β-cell function, measured as oral disposition index obtained during an OGTT, but not associated with an increase in β-cell mass compared to control (107). In humans, Derosa *et al.* (108) investigated the effect of a DPP-4 inhibitor plus metformin compared to metformin plus placebo on the secretory capacity of β-cells using euglycemic hyperinsulinemic and hyperglycaemic clamps combined with subsequent arginine stimulation. They found an improved β-cell function, expressed as disposition index, after 12 months of DPP-4 treatment (from 163.8 ± 37.9 to 279.5 ± 56.9 nmol/L×µmol/kg) compared to control (from 163.6 ± 37.7 to 214.2 ± 48.4 nmol/L×µmol/kg).

Although the effects of DPP-4 inhibitors are mainly thought to be via increased incretin levels, Aulinger *et al.* (109) studied the effect of DPP-4 inhibitors on glucose homeostasis in patients with T2D after blocking GLP-1 action through a GLP-1 receptor antagonist. Interestingly, they found a significant effect of DPP-4 inhibitor on insulin secretion during an OGTT despite GLP-1 receptor blockade. Increased GIP action is a possible candidate to explain this independent effect. Yanagimachi *et al.* (110) measured incretin levels after DPP-4 inhibitor administration during an OGTT in non-diabetic subjects, and found that DPP-4 administration not only increased GLP-1 but also bioactive GIP levels.

5.3.2. DPP-4 inhibitors and insulin sensitivity

DPP-4 inhibitors' effect on insulin sensitivity have been investigated in animal models. For instance, in rats, Pospisilik *et al.* (111) found an increase in insulin-mediated glucose uptake in muscle tissue as well as an increase in insulin sensitivity measured by the Matsuda index upon DPP-4 inhibitors compared to control. Nevertheless, in humans, the effect of DPP-4 inhibitors on insulin sensitivity remains controversial. Derosa *et al.* (112) evaluated the effect of a DPP-4 inhibitor as add-on therapy on insulin sensitivity in subjects with T2D and found that HOMA-IR significantly decreased after 12, 18 and 24 months of treatment in the intervention group compared to the control group. However, HOMA-IR does not accurately measure insulin sensitivity in interventions studies. Parthan *et al.* (113) found no effect of 6 months of DPP-4 inhibitor treatment compared to placebo on insulin sensitivity, as measured by hyperinsulinemic-euglycemic clamp, in well-controlled T2D subjects. These results suggest

that, despite a reduction in HbA1c and fasting glucose levels, there seems to be a lack of effect of DPP-4 inhibitors on insulin sensitivity, which contrasts the effects of GLP-1RA interventions. A possible explanation may be the fact that DPP-4 inhibitors in several studies does not seem to have any significant effect on weight loss (106, 114).

Interestingly, in animal models of obesity, weight gain has been associated with an increase in DPP-4 expression in hepatic tissue (115). In humans, DPP-4 activity has also been associated with a higher BMI, increased fat percentage and NAFLD (116). These findings may suggest that DPP-4 inhibition would be a target to reduce hepatic fat content. Indeed, DPP-4 inhibitor treatment in animal models has been associated with improvements in liver steatosis (117, 118) as well as in fibrosis (119). However, in humans treatment with DPP-4 inhibitors has not shown any effect on NAFLD (120, 121).

In conclusion, DPP-4 inhibitors have a significant effect on insulin secretion compared to placebo and probably their main effect on glucose control is via increasing insulin secretion rather than having an effect on insulin sensitivity. DPP-4 inhibitors when compared to GLP-1RA treatment seem to have no effect on body weight, and may therefore be less favourable for patients that mainly benefit from weight loss. We suggest that DPP-4 inhibitors therapy could be a treatment option for SIDD and MARD because of the lack of DPP4 inhibition on body weight and insulin resistance.

5.4. Thiazolidinedione

Thiazolidinediones, also known as glitazones, belong to the group of insulin sensitizers. Thiazolidinediones were first discovered by screening for a hypoglycaemic effect in ob/ob mice (122). Later, it was discovered that thiazolidinediones improved insulin sensitivity in insulin resistant animal models. In humans, similar results are found, as thiazolidinedione administration causes a reduction in glucose and insulin levels, improves insulin resistance and improves lipid metabolism. It is generally accepted that thiazolidinediones act as a nuclear peroxisome proliferator-activated receptor (PPAR) agonist, specifically for the gamma subtype (PPAR-γ), which is predominantly expressed in white adipose tissue, but to a lesser extend also in muscle, liver and heart (123, 124). Activation of PPAR-γ results in transcription of the PPARγ target genes, which are mainly involved in lipid and carbohydrate metabolism and immune functions (125-127). Due to severe adverse effects, most types of thiazolidinediones, including troglitazone and rosiglitazone, have been withdrawn from the market. Only pioglitazone is currently approved by both the authorities of Europe (EMA) and the United States of America (FDA) for treatment of T2D, and we therefore focus solely on this thiazolidinedione in this review. In general, pioglitazone administration is associated with plasma glucose reduction of 1.2 - 2.0 mmol/L and a HbA1c reduction of 0.9 - 1.3%, and an increase in body weight of 3.6 kg (128, 129).

5.4.1. Thiazolidinediones and β-cell function

The effect of pioglitazone on β-cell function has been established in a meta-analysis (130). With monotherapy, HOMA-B improved by 16% compared to baseline. When pioglitazone was combined with metformin or sitagliptin (a DPP-4 inhibitor) a small but significant improvement of 9.8 and 11.8% in HOMA-B, respectively, was observed in patients with T2D. However, although HOMA-B provides some information about the effect of pioglitazone on β-cell function, trials using the gold standard to assess β-cell function, the disposition index, are limited. To the best of our knowledge, there are only 2 clinical trials who reported β-cell function assessed via the disposition index in patients with T2D. Gastaldelli *et al.* (131) and Tripathy *et al.* (132) reported an improved β-cell function measured as disposition index upon pioglitazone administration for 4 and 6 months, respectively. How pioglitazone would improve β-cell function is unknown, but it may involve direct (expression of PPAR-γ in pancreatic islets cells (133)) or indirect effects related to the marked improvements in insulin sensitivity by pioglitazone (see below).

Over a longer period of time, measured in the PROactive trial with an average follow up of 34.5 months, pioglitazone was more effective in reducing HbA1c levels than placebo in patients treated with either metformin of sulfonylureas. The reduction in HbA1c occurred rapidly and was sustained over the full period of time (134) indicating a long-term effect of pioglitazone on preserving β-cell function.

5.4.2. Thiazolidinediones and insulin sensitivity

The effects of thiazolidinediones on insulin sensitivity in humans has extensively been studied and reviewed over the years. In a systematic review, Natali and Ferrannini (135) identified 23 papers which measured the effect of thiazolidinediones on peripheral glucose disposal by the hyperinsulinemic clamp and/or EGP using glucose tracer analysis in patients with T2D. A combined data analysis revealed improvements in the range of 31 - 36% and 19 - 33% in peripheral and hepatic insulin sensitivity, respectively, upon thiazolidinedione administration compared to baseline or placebo. However, it should be noted that in this systematic review not only pioglitazone was included, but also troglitazone and rosiglitazone.

With respect to pioglitazone, several research groups showed a statistically significant improved peripheral (131, 136-146), hepatic (131, 143-146) and adipose tissue (137, 145, 147) insulin sensitivity in patients with T2D.

Because PPARγ is predominantly expressed in adipose tissue, it is suggested that the improvements in peripheral and hepatic insulin sensitivity as well as in β-cell function are indirect, mainly elicited by a decreased flux of fatty acids from adipose tissue thereby increasing insulin-mediated glucose uptake and reducing lipotoxicity. It is well known that

PPARγ activation by pioglitazone leads to reduced plasma levels of triglycerides and FFA (148, 149). Since higher FFA levels are associated with ectopic fat accumulation and insulin resistance, the lowering of FFA probably play an important role for the improvement in insulin sensitivity. Indeed, pioglitazone administration is associated with a redistribution of adipose tissue, resulting in reduced ectopic and visceral lipid storage, but an increase in subcutaneous adipose tissue. Promrat *et al.* (150) were the first to describe the effects of pioglitazone administration on hepatic lipid content in non-diabetic patients with NASH. In this trial, hepatic lipid content decreased significantly from 47.5% to 22.8% after 48 weeks of pioglitazone administration, but total body fat percentage increased from 35.8% to 37.6%. At the same time, the insulin sensitivity index, assessed during a frequently sampled intravenous glucose tolerance test, improved. Rasouli *et al.* (140) investigated the effects of pioglitazone versus metformin administration for 10 weeks on insulin sensitivity and intramyocellular lipid content (IMCL) in patients with IGT. They reported a significant decrease in IMCL upon pioglitazone compared to metformin therapy and compared to baseline. This lowering of IMCL content was accompanied by an increase in insulin sensitivity, assessed via an insulin-modified intravenous glucose tolerance test, and with a redistribution of visceral fat towards subcutaneous fat stores.

Similar results were later reported by several other research groups Thus, pioglitazone administration in patients with prediabetes and T2D, co-treated with dietary advice, hypocaloric diet, metformin or insulin led to a decrease in hepatic (145, 151-154), intramyocellular (152) and myocardial (154) lipid content, but also to an increase in subcutaneous fat (145, 152, 154). In spite of reduced ectopic fat, treatment results in an increase in body weight. This increase in body weight is the result of a higher caloric intake in patients treated with pioglitazone (155).

It should be noted that not all studies are consistent in the effect of pioglitazone on metabolic adaptations. Thus, Phielix *et al.* (147) did report an improved adipose tissue insulin sensitivity but did not find an improved peripheral or hepatic insulin sensitivity, despite a decrease in hepatic lipid content upon 12 weeks of pioglitazone therapy in non-obese patients with T2D. In addition, van der Meer *et al.* (156) reported a decreased hepatic lipid content, but no changes in intramyocardial lipid content or myocardial fatty acid oxidation upon 24 weeks of pioglitazone administration in patients with T2D. Bajpayi *et al.* (136) reported a significant shift from IMCL towards extramyocellular lipid (EMCL) in the gastrocnemius, tibialis anterior and soleus muscles, and a tendency towards a decrease in hepatic lipid content after 12 weeks of pioglitazone administration in patients with T2D. These changes were accompanied with an improved peripheral insulin sensitivity and metabolic flexibility (ΔRespiratory quotient) measured during the insulin infusion (80 mU/min/m^2) compared to fasted state of a hyperinsulinemic-euglycemic clamp. Substrate oxidation in the fasted state and mitochondrial function, assessed as resting ATP turnover and maximal ATP synthetic rate by ^{31}P-MRS were unaffected by pioglitazone.

In conclusion, pioglitazone is effective in reducing peripheral, hepatic and adipocyte insulin resistance, mainly by amelioration of lipotoxicity by reducing ectopic lipid storages. Pioglitazone is also effective in lowering HbA1c over a longer period, reflecting an improved β-cell function. However, these effects are not sustained after discontinuation of pioglitazone (157). Pioglitazone can be a powerful treatment in a limited group of patients, where improvements in insulin resistance and NAFLD outweigh the side effects like weight gain, osteoporosis (158) and water retention increasing the risk for heart failure (159). We hypothesise that pioglitazone could be a beneficial treatment for SIDD and SIRD and should be avoided in patients with MOD and MARD due to the side effects.

6. CONCLUDING REMARKS

T2D is a heterogeneous disease with a complex metabolic disarray leading to hyperglycaemia and progressive β-cell dysfunction. Currently, several second-line treatment options exist, however, finding the most optimal type of medication for a patient with T2D can be challenging. The classification system suggested by Ahlqvist *et al.* provides a spectrum of the disease that provides more insight to the underlying metabolic cause of T2D.

Based on the reported effects of the current available antidiabetic medications on β-cell function, insulin sensitivity and metabolism, some medications may be more suitable to treat subgroups of patients. Metformin is the recommended first line therapy for glucose control of patients with T2D and possibly works as a first line therapy for patients belonging to all four T2D subgroups. Metformin may even be sufficient as the only therapy in patients with mild disease mainly, including some patients with MARD and MOD. However, for patients with severe insulin-deficiency (SIDD), we conclude that these patients could benefit from most of the second-line current antidiabetic treatments. Since the SIDD group is associated with a lower BMI, there is also no preferred type of medication for those patients to correct body weight.

Patients with severe insulin resistance (SIRD), characterized by a higher BMI and a higher prevalence of NAFLD, may benefit most from treatments that reduce body weight and improve insulin sensitivity. This group of medications would include SGLT2 inhibitors because of the potential improvements in insulin sensitivity and clinically relevant reductions in body weight. Whether GLP-1RA treatment would be beneficial in this group is still unclear due to the limited clinical trials investigating insulin sensitivity. However, as GLP-1RA reduces body weight and hepatic lipid content, this treatment option could be beneficial for SIRD. Treatment with pioglitazone is also effective in improving insulin sensitivity and reducing NAFLD, but should only be considered when there are no other treatment options available because of the well-established weight gain and other adverse effects associated with

pioglitazone administration. DPP-4 inhibitors do not seem to have a therapeutic role in this group as there are no established effects on insulin sensitivity, weight reduction or NAFLD.

Patients with mild-obesity related diabetes (MOD) are characterized by mild insulin resistance and mild insulin deficiency, but a high BMI. This particular group may already benefit from treatment with metformin only, but if this treatment is insufficient to control glucose levels they could benefit the most from GLP-1RA and SGLT2 inhibitor treatment, as both medications reduce body weight significantly. Treatment with pioglitazone should be avoided in this group, because of the weight gain associated with this medication.

For patients with mild age-related diabetes (MARD), who are characterized by mild insulin resistance and mild insulin deficiency, higher age at the time of the diagnosis, and lower risk for developing end-organ damage, sulfonylurea and DPP-4 inhibitors could be the best options as additional therapies if metformin treatment alone does not result in glucose control. However, SGLT2 inhibitors and GLP-1RAs may also be an option in MARD patients with established end-organ diseases like CV disease and reduced kidney function. Since the age in this population is higher, add-on treatment decisions should be made carefully and possible side effects of each type of medication should – as always - be considered.

In order to achieve adequate therapy in T2D, we have here considered that an important proportion of patients will require additional medication on top of lifestyle recommendations and metformin treatment. The subgroups proposed by Ahlqvist *et al.* and the known metabolic effects on β-cell function and insulin sensitivity of the different classes of medication may help to provide a more personalized treatment of patients with T2D, based on the main underlying cause of hyperglycaemia in each individual, as outlined above. However, the final treatment choice in T2D patients should also include consideration of other aspects associated with diabetes. For example, in the presence of CV disease, a GLP-1RA or SGLT2 inhibitor are the preferred options without considering their belonging to subgroups. Other factors such as the presence of kidney disease, the importance of weight loss in combination with lifestyle interventions, patient age and preferences and potential side effects should be weighted too.

Finally, we are aware that the Ahlqvist clustering of subgroups taking the metabolic phenotype of T2D into account may not be the final diabetes classification and more investigations are needed. Furthermore, there are currently no intervention trials performed to provide the scientific evidence which antidiabetic medication is best for patients depending on their metabolic phenotype. Therefore, the suggestions described above are hypothesise generating, and should not be regarded as recommendations. To establish the most appropriate therapy, future intervention trials are needed in diabetes subgroups in order to provide a scientific basis to develop personalized medicine for treatment of the large and diverse group of patients with T2D.

AUTHOR CONTRIBUTIONS

All authors contributed in writing the manuscript and approved the final version.

DECLARATION OF INTEREST

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CHAPTER 3

EMBARGOED

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

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Submitted for publication

CHAPTER 4

EMBARGOED

One night of prolonged fasting does improve overnight substrate oxidation, without modulating hepatic glycogen in individuals with NAFL and healthy age-matched individuals: a randomized cross-over trial

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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

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. Randomization was performed by a study-independent investigator, ensuring that an equal amount or 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. Fourteen adults with type 2 diabetes (BMI 30.5±4.2 kg/m^2, HbA1c 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. 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.

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 supplementary 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 measured 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 (determined 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 Corp, 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.

Table 1 Baseline characteristics of participants

Characteristic	Measurement/value
N	14
Sex, *n* female/*n* male	7/7
Age, years	67.5±5.2
BMI, kg/m^2	30.5±3.7
Diabetes medication, *n* yes/*n* no	10/4
Metformin only, *n*	7
Metformin + gliclazide, *n*	3
Fasting plasma glucose, mmol/l	7.9±0.4
HbA_{1c}, mmol/mol	46.1±7.2
HbA_{1c}, %	6.4±0.7
AST, μkat/l	0.4±0.1
ALT, μkat/l	0.4±0.2
GGT, μkat/l	0.5±0.2
eGFR, ml min^{-1} 1.73 m^{-2}	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

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).

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 16.4±1.3 vs 16.1±1.3%, p=0.57; mono-unsaturated fatty acids 40.8±1.0 vs 43.3±1.5%, 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=0.64). However, absolute levels of NEFAs were lower with TRE during the low- and high-insulin phase (p=0.01 and p=0.04; Fig. 1b), which may hint at an improved adipose tissue insulin sensitivity.

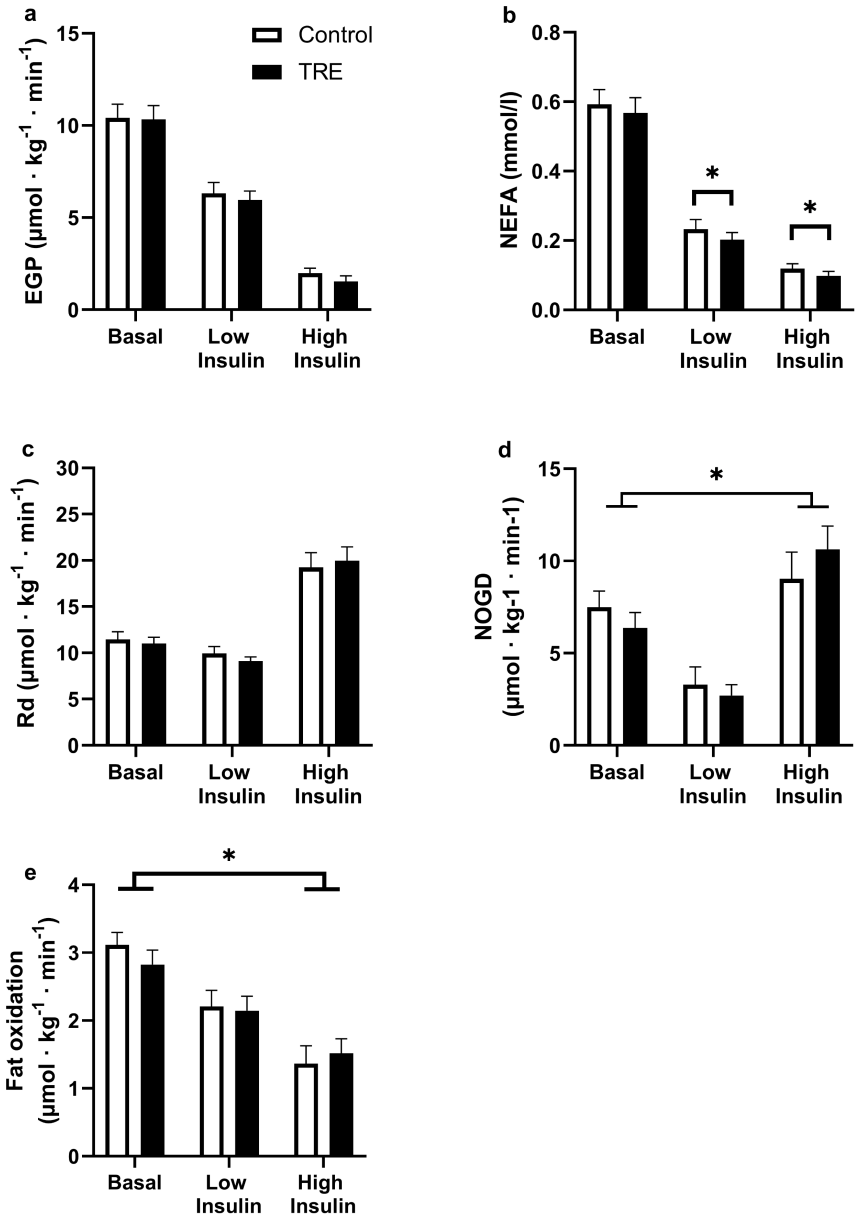

Figure 1: Effect of TRE on EGP (a), plasma NEFA (b), R_d (c), NOGD (d) and fat oxidation (e) measured during a hyperinsulinaemic–euglycaemic two-step clamp (n=14). *p < 0.05 (data were analysed with paired t-tests)

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–e, 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).

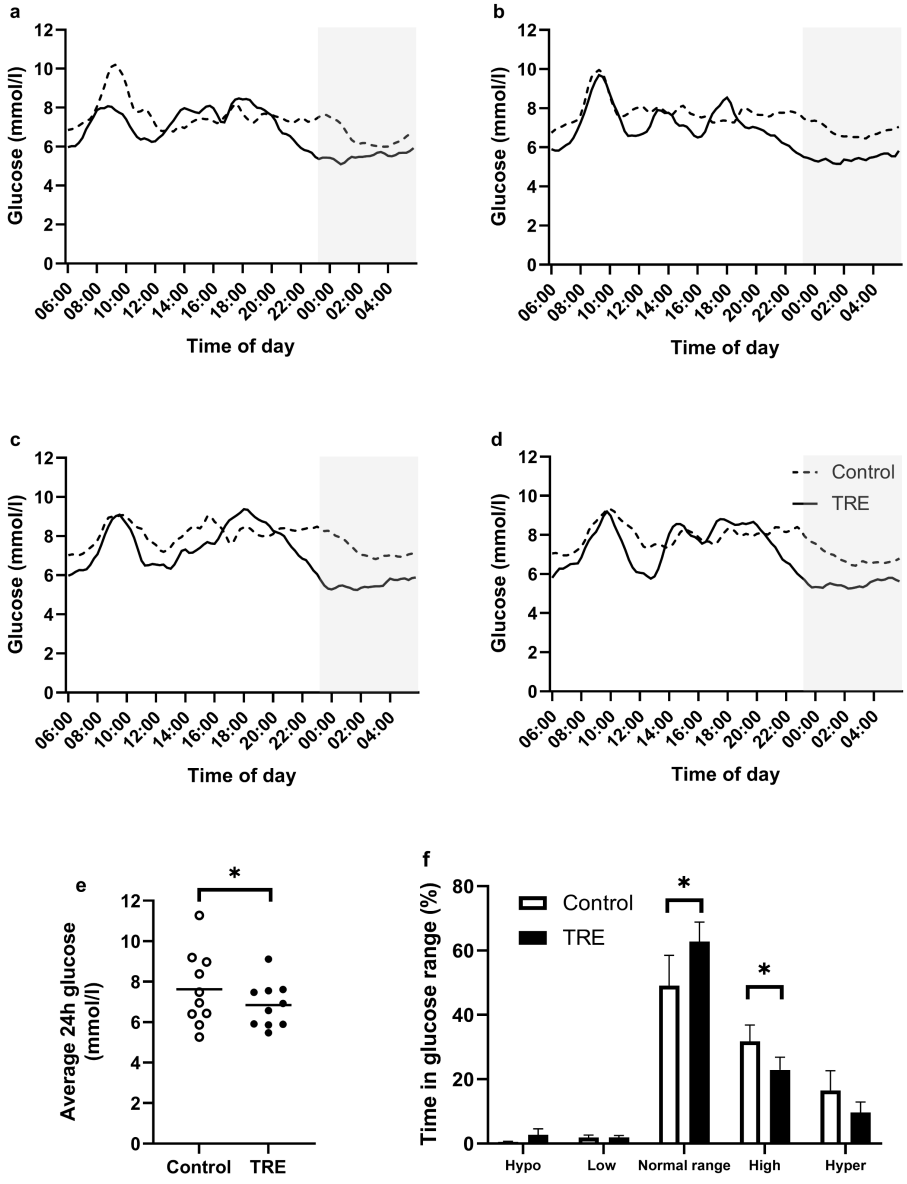

Figure 2: Twenty-four-hour glucose levels on days 15 (a), 16 (b), 17 (c) and 18 (d) during TRE or CON (n=10). Mean 24 h glucose from day 15 to day 18 (n=10) analysed using a paired t test (e). Time spent in glucose range during days 15–18 (n=10) analysed using Wilcoxon tests with Bonferroni correction (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, mU/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

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=0.81; 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=0.16; 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).

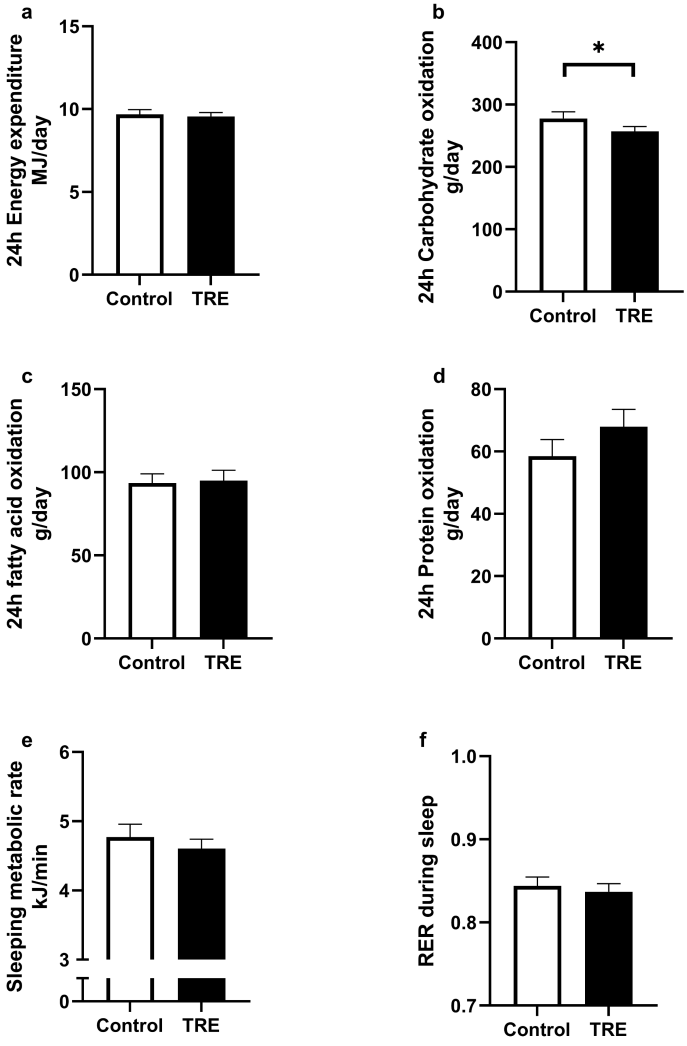

Figure 3: Effect of TRE on 24 h energy expenditure (**a**), substrate oxidation (**b – d**), sleeping metabolic rate (**e**), and RER during sleep (**f**) (*n*=13). *$p<0.05$

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, glutamate; 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 14 h. 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.

ACKNOWLEDGEMENTS

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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

The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement

CA, CF, JH and PS designed the experiments. CA, CF, 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 ^{1}H-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-[6,6-2H2] glucose to determine baseline endogenous glucose production (EGP), glucose appearance (Ra) and glucose disposal (Rd). Afterwards, insulin was infused at 10 mU · m2 · min-1 to assess hepatic insulin sensitivity reflected by suppression of EGP. After 3 h, insulin infusion was increased to 40 mU · m2 · 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 R_a and R_d [10].

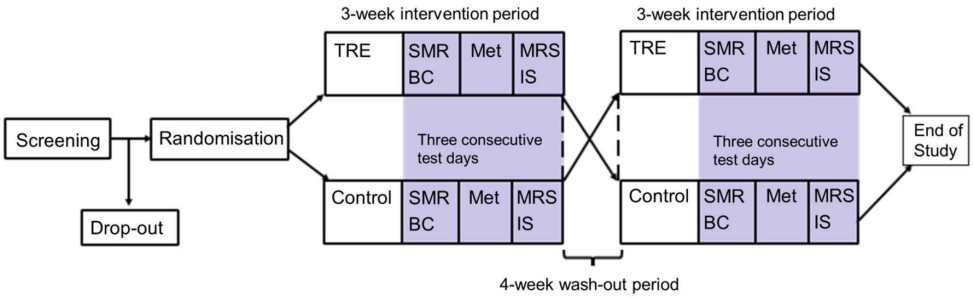
Sample size was determined based on the variability in glycogen content measured in the fasted state with ^{13}C-MRS, which amounted 7 – 11% in previous studies [11, 12]. For a more conservative estimation of the standard deviation, 11% was used in our calculation. The following equation was used for sample size calculation: $N = \sigma 2/\Delta\mu 2 * (Z0.8 + Z0.975)2$ with $Z0.8 = 0.842$, $Z0.975 = 1.960$, $\sigma 2 = 11\%$, 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

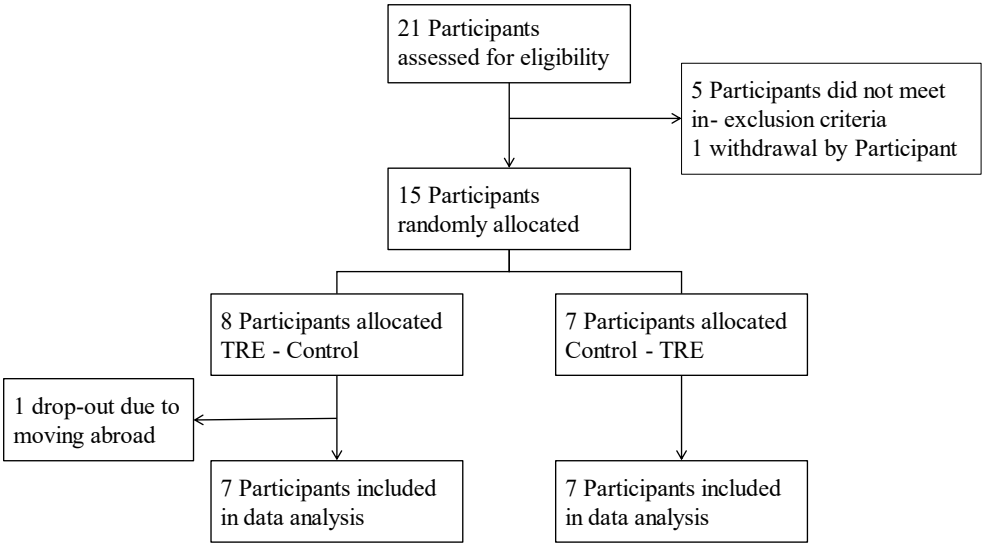
ESM Table 1: Inclusion criteria of the trial

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 cotransporter-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

Impact of age and BMI on nocturnal substrate oxidation: a combined data analysis

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In preparation

ABSTRACT

Background

Twenty-four-hour substrate metabolism is characterized by high levels of carbohydrate oxidation in the postprandial state and high levels of fat oxidation in the overnight fasted state. Recently, we showed that in older metabolically compromised individuals this 24 hour eating-fasted cycle in substrate oxidation is blunted, with lower rates of nocturnal fat oxidation and higher rates of nocturnal carbohydrate oxidation compared to healthy young lean individuals. As these populations were not matched for age, BMI and other parameters such as fasting glucose, it is unclear if participant characteristics can predict this lack of switch in substrate oxidation during the night. More specifically, we investigate whether baseline participant characteristics can explain the differences observed in nocturnal substrate oxidation between different populations.

Methods

Baseline data from the placebo or control arm of 10 human clinical trials were combined, resulting in a total of 124 participants, divided into 23 young lean individuals, 10 older lean individuals, 44 older individuals with overweight or obesity, but without type 2 diabetes, and 47 older individuals with overweight or obesity and type 2 diabetes. Linear mixed model was used to detect differences between populations in nocturnal respiratory exchange ratio and substrate oxidation. Pearson's correlation coefficient analysis was performed to identify which baseline characteristics influence nocturnal respiratory exchange ratio (RER) and substrate oxidation.

Results

Nocturnal RER was the highest in the overweight and type 2 diabetes individuals compared to both the young and old lean individuals ($p < 0.05$ for both). In line with the RER, nocturnal carbohydrate oxidation was highest, and nocturnal fat oxidation was lowest in these individuals. Baseline BMI correlated with nocturnal RER and fat oxidation ($r = 0.259$ and $r = -0.559$), with a higher BMI associated with higher nocturnal RER and lower nocturnal fat oxidation.

Conclusion

Being overweight, with or without type 2 diabetes, is the main driver of high nocturnal carbohydrate and low fat oxidation. Age per se does not lead to changes in nocturnal substrate oxidation compared to young, lean participants. BMI negatively correlated with nocturnal fat oxidation, indicating that BMI, or BMI-associated metabolic alterations, is related to alterations in nocturnal substrate oxidation.

INTRODUCTION

Metabolic health is characterized by having good metabolic flexibility with high rates of carbohydrate oxidation during the daytime when there is carbohydrate intake and high rates of fat oxidation during the nocturnal period when there is no carbohydrate intake (1). During daytime carbohydrate intake results in the release of insulin by the pancreatic β-cells. Insulin acts on many different organs and pathways, resulting in the uptake of carbohydrates by skeletal muscle, liver, and adipose tissue, as well as the inhibition of lipolysis by the adipose tissue. Together, this results in higher whole-body carbohydrate oxidation and lower fat oxidation. During the night-time, insulin levels drop while glucagon levels rise, when there is no carbohydrate intake. Glucagon has opposite effects of insulin, stimulating lipolysis by adipose tissue, resulting in higher fat oxidation rates.

Recently we showed that older metabolically compromised individuals have however lower rates of nocturnal fat oxidation and higher rates of nocturnal carbohydrate oxidation compared to healthy young lean individuals (2, 3), whereas during daytime no major differences in substrate oxidation were observed. This indicates that older metabolically compromised individuals do not reach a true nocturnal fasted state, even when activities and food intake during the day preceding the night are similar and controlled. This raises the question why metabolically compromised individuals have higher nocturnal carbohydrate oxidation rates compared to young lean individuals. A major confounder in these studies was that the older metabolically compromised individuals were not only older, but also were overweight or obesity, and had slightly higher glucose levels compared to the young lean individuals.

It is already known that individuals with overweight and obesity, insulin resistance, as well as non-alcoholic fatty liver disease (NAFLD) are characterized by an impaired metabolic inflexibility, as determined during a hyperinsulinaemic–euglycaemic clamp or a meal test (4-7). However, separating these factors is difficult, as obesity, insulin resistance, and NAFLD are interrelated, and therefore it is unclear which characteristic is responsible for metabolic inflexibility. How these factors influence nocturnal substrate oxidation has not been studied extensively either.

Therefore, we here aim to unravel whether baseline participant characteristics can explain the differences observed in nocturnal substrate oxidation between populations. To this end, we combined baseline data from the placebo arm or control arm of 10 human clinical trials with a comparable study design and investigated which participant characteristic contributes most to the variation in nocturnal substrate oxidation.

METHODS

Participants and study design

Data from 10 human clinical trials were combined. All clinical trials were approved by the Ethics Committee of Maastricht University Medical Center and were registered at clinicaltrials.gov (NCT02261168, NCT02835664, NCT03338855, NCT03593343, NCT03721874, NCT03733743, NCT03800290, NCT03992248, and NCT04510155) or trialregister.nl (NTR7426). The combined clinical trials resulted in a total of 124 participants, divided into 23 young lean individuals (age between 18 – 30 years, BMI < 25, referred to as young lean), 10 older lean individuals (age between 40 – 75, BMI < 25, referred to as old lean), 44 older individuals with overweight or obesity, but without type 2 diabetes (age between 40 – 75, BMI ≥ 25, referred to as overweight), and 47 older individuals with overweight or obesity and type 2 diabetes (age between 40 – 75, BMI ≥ 25, and diagnosed with type 2 diabetes, referred to as type 2 diabetes). Depending on the set-up of the clinical trial, data were obtained from either the screening or baseline measurement or at the end of the placebo-controlled or control condition.

Indirect calorimetry

Oxygen consumption and carbon dioxide production were continuously measured during the night using whole-chamber indirect calorimetry [Omnical, Maastricht Instruments, Maastricht, the Netherlands (8)]. From the indirect calorimetry data, energy expenditure and substrate oxidation were calculated using the Brouwer equation (9). Protein oxidation is estimated to be 12.4% of the total energy expenditure using the Weir equation (10). The respiratory exchange ratio (RER) was calculated by dividing carbon dioxide production by oxygen consumption. Energy expenditure, RER, and substrate oxidation were calculated over the whole night (0.30 – 5 am), and during three parts of the night (0.30 – 2 am, 2 – 3.30 am, and 3.30 – 5 am) to assess the pattern in substrate oxidation over time.

Hepatic lipid content

Hepatic lipid content was quantified by proton magnetic resonance spectroscopy (^{1}H-MRS) on a 3T MRI-scanner (Achieva 3T-X, Philips Healthcare, Best, The Netherlands). Liver fat spectra were acquired by either 2 different protocols, as described previously (11, 12). Lipid content values are given as a T2 corrected ratio of the CH_2 peak relative to the sum of the unsuppressed water peak and the CH_2 peak, converted to weight/weight percentage (13, 14). Depending on the clinical trial, spectra were acquired in the overnight fasted state, or after ≥ 3 hours of fasting.

Statistics

Results are presented as means ± SEM. First, differences between four populations were tested by one-way ANOVA combined with Tukey's post hoc test. In the second analysis, differences between populations in nocturnal energy expenditure, RER, and substrate oxidation were tested using the linear mixed model (LMM) procedure. The LMMs included fixed effects for population (young lean, old lean, overweight, and type 2 diabetes). In a separate analysis, also the fixed effects of time (0.30 – 2 am, 2 – 3.30 am, 3.30 – 5 am) and sex (male and female) were added, including interaction effects. When statistical differences were observed, pairwise comparison was performed with Bonferroni-correction for multiple comparisons. In the third analysis, Pearson's correlation coefficient analysis was performed to identify which baseline characteristics influence nocturnal RER and substrate oxidation. Analyses were performed for n = 124 unless specified otherwise. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 27.0.

RESULTS

Baseline characteristics among different populations

In total, we included 124 participants, divided into 23 young lean, 10 old lean, 44 overweight, and 47 type 2 diabetes participants per population. By design, the young lean population was younger than the other populations ($p < 0.001$ between groups). In addition, the young lean and old lean population had a lower BMI compared to the overweight and type 2 diabetes population ($p < 0.001$ between groups). The overweight population consisted of more females compared to the young lean population ($p = 0.018$ between groups), since the young lean population consisted of only males. Furthermore, the type 2 diabetes population had higher fasting plasma glucose levels compared to the other three populations ($p < 0.001$ between groups), and a higher liver fat percentage compared to the young lean and old lean populations ($p < 0.001$ between groups). Dinner time, which was determined by the study protocols, was earlier in the old lean population ($p < 0.001$ between groups). All individual values can be found in table 1.

Table 1. Baseline differences between populations

Variable		Total	Populations				P-values between groups
			Young lean	Old lean	Overweight	Type 2 diabetes	
Sex	F (%)	31 (25.0)	0 (0.0) [a]	3 (30.0) [a, b]	15 (34.1) [b]	13 (27.7) [a, b]	0.018
	n	124	23	10	44	47	
Age	year	56.7 ± 1.5	23.5 ± 0.7 [a]	64.9 ± 2.2 [b]	63.1 ± 1.1 [b]	65.2 ± 0.8 [b]	<0.001
	n	124	23	10	44	47	
BMI	kg/m²	27.8 ± 0.4	22.8 ± 0.4 [a]	23.7 ± 0.6 [a]	30.2 ± 0.4 [b]	29.0 ± 0.5 [b]	<0.001
	n	124	23	10	44	47	
Glucose	mmol/L	6.7 ± 0.2	5.3 ± 0.2 [a]	5.3 ± 0.1 [a]	5.7 ± 0.1 [a]	8.3 ± 0.2 [b]	<0.001
	n	111	11	10	44	46	
Liver fat	%	6.6 ± 0.8	0.6 ± 0.2 [a]	1.3 ± 0.4 [a]	5.9 ± 0.9 [a, b]	10.0 ± 1.5 [b]	<0.001
	n	93	11	10	31	41	
Dinner time	hh.mm	18.04 ± 0.03	18.14 ± 0.03[a]	16.30 ± 0.00 [b]	18.07 ± 0.07[a]	18.16 ± 0.02[a]	<0.001
	n	124	23	10	44	47	

Data are presented as mean ± SEM. Numbers followed by different letters are statistical differences between populations ($p \leq 0.05$ (Tukey's test)).

Nocturnal energy expenditure and substrate oxidation among different populations

To assess differences in nocturnal substrate oxidation among the populations, LMM was used with a fixed effect for populations. First, we assessed nocturnal RER, which reflects the relative contribution of carbohydrate and fat oxidation. RER during the full night was significantly different between populations (population: $p < 0.001$). The RER of the young lean and old lean populations was statistically lower compared to the overweight and type 2 diabetes populations ($p < 0.05$ for both, figure 1A). No differences were observed between the young lean and old lean population ($p=1.000$), nor between the overweight and type 2 diabetes population ($p = 1.000$). These results suggest that carbohydrate oxidation is higher and fat oxidation lower in overweight and type 2 diabetes patients.

To test if differences in RER were due to differences in energy expenditure, we compared energy expenditure between populations. Energy expenditure was significantly different between the populations (population: $p = 0.002$, figure 1C). Energy expenditure of the old lean population was statistically lower compared to the overweight and type 2 diabetes population ($p = 0.019$ and $p < 0.001$, respectively), and tended to be lower compared to the young lean population ($p = 0.069$). No differences were observed between the other populations.

Because of the differences in energy expenditure, we also examined absolute carbohydrate and fat oxidation, expressed per kg body weight. Carbohydrate oxidation was significantly different between the populations (population: $p = 0.007$, figure 1D), with significantly higher carbohydrate oxidation in the type 2 diabetes population compared to the young lean and old lean populations ($p = 0.030$ and $p = 0.044$, respectively). Although carbohydrate oxidation of the overweight population seemed to be higher than in the young lean and old lean populations, there was no statistical difference ($p = 0.845$ and $p = 0.528$, respectively). No differences were observed between the other populations. Furthermore, nocturnal fat oxidation was also significantly different between the population (population: $p < 0.001$, figure 1F). Fat oxidation was lower in the overweight and type 2 diabetes population compared to the young lean and old lean population ($p < 0.01$). No differences were observed between the young lean and old lean, and between the overweight and type 2 diabetes population. In summary, our analysis shows that the differences in substrate oxidation, expressed per kg body weight, between the populations were characterized by lower fat oxidation in the overweight and type 2 diabetes population, and by higher carbohydrate oxidation in type 2 diabetes patients, as was also reflected in the differences observed in RER between these populations.

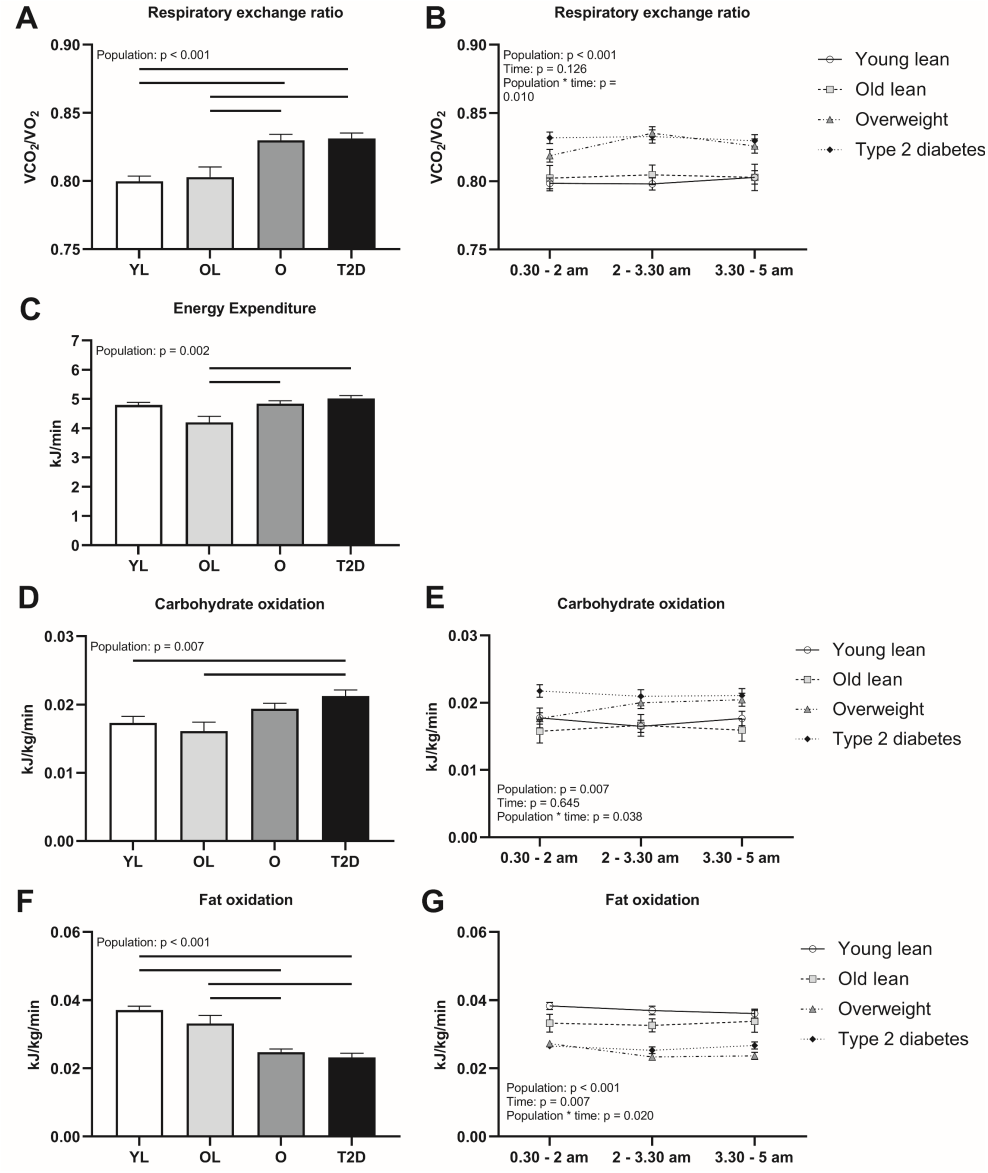

Figure 1: Nocturnal respiratory exchange ratio, energy expenditure, and substrate oxidation during the night in different populations. A: Overnight respiratory exchange ratio in the different populations; **B**: Respiratory exchange ratio over the course of the night in the different populations; **C**: Overnight energy expenditure in the different populations; **D**: Overnight carbohydrate oxidation in the different populations; **E**: Carbohydrate oxidation over the course of the night in the different populations; **F**: Overnight fat oxidation in the different populations; **G**: Fat oxidation over the course of the night in the different populations. For the young lean population, n = 23; for the old lean population, n = 10; for the overweight population, n = 44; for the type 2 diabetes population, n=47. Data are presented as mean ± SEM. Lines indicate significant differences between populations. O, overweight; OL, old lean; T2D, type 2 diabetes; YL, young lean.

As it is generally thought that the contribution of carbohydrate oxidation decreases and fat oxidation increases during the night, we also assessed the effect over time. To assess this pattern LMM was used with fixed effects for population and time (i.e., 0.30 – 2 am, 2 – 3.30 am, and 3.30 – 5 am), and an interaction effect (population*time). Nocturnal RER exhibited different patterns among the populations (population*time: p = 0.010, figure 1B). RER of the young lean, old lean, and type 2 diabetes population remained stable during the night (p = 1.000 for all). In contrast, the RER of the overweight population is statistically lower in the first part of the night compared to the middle and latest part (p < 0.001), while no differences were observed from the middle part of the night compared to the latest part. In line with the RER, both carbohydrate oxidation and fat oxidation exhibited different patterns during the night among the populations (p = 0.038 and p = 0.020, respectively, figure 1E and 1G). In the overweight population carbohydrate oxidation was higher and fat oxidation lower in the first part of the night compared to the middle and latest part (p < 0.05), while no differences were observed from the middle part of the night compared to the latest part, or in the other populations.

Baseline characteristics and nocturnal substrate metabolism

Next, we examined whether baseline characteristics correlated with nocturnal RER and substrate oxidation. Nocturnal RER correlated positively with age (r = 0.399, p < 0.001, figure 2A) and BMI (r = 0.259, p = 0.004, figure 2B). Although age correlated positively with nocturnal RER, it can be seen from figure 2A that this is mainly driven by the young population, which is very separate from the other participants with respect to age. Indeed, if the young individuals were removed from analysis, no correlation between age and nocturnal RER was observed (r = 0.150, p = 0.135). Furthermore, nocturnal RER tended to correlate with fasting glucose (r = 0.161, p = 0.092, figure 2C), but this did not reach statistical significance. Nocturnal RER did not correlate with liver fat percentage (r = 0.055, p = 0.601, figure 2D). Nocturnal carbohydrate oxidation, expressed per kg body weight, did correlate positively with age (r = 0.221, p = 0.013, figure 2E), and tended to correlate with fasting glucose (r = 0.172, p = 0.071, figure 2G). No correlation was observed between nocturnal carbohydrate oxidation and BMI or liver fat percentage (BMI: r = -0.023, p = 0.804, figure 2F; liver fat: r = -0.014, p = 0.893, figure 2H). Nocturnal fat oxidation, expressed per kg body weight, did correlate negatively with age and positively with BMI (Age: r = -0.554, p < 0.001, figure 2I; BMI: r = 0.559, p < 0.001, figure 2J). Nocturnal fat oxidation tended to correlate negatively with fasting glucose (r = -0.173, p = 0.070, figure 2K), but did not reach statistical significance. No correlation was observed between fat oxidation and liver fat percentage (r = -0.142, p = 0.174, figure 2L). Again, correlations with age were no longer present when the young individuals were removed from the analysis (carbohydrate oxidation: r = 0.177, p = 0.076; fat oxidation: r = -0.082, p = 0.413)

We also assessed whether RER, carbohydrate oxidation, and fat oxidation were also different between males and females. To this end, we again performed LMM and added an additional variable of "sex", as well as an interaction variable (population*sex). We found that males and females tended to have a different nocturnal RER across the populations (population*sex, p = 0.071, figure 3A), with a higher RER in males compared to females in the overweight population (p = 0.026). No differences in RER in the different sexes were observed in the other populations. Carbohydrate oxidation was not different between the sexes and between the population (population *sex, p = 0.220, figure 3B). Fat oxidation tended to be different in males and females but did not reach statistical significance (population*sex, p = 0.067, figure 3C). In the old lean population, males tended to have higher fat oxidation compared to females (p = 0.087), although it should be noted that this population consisted of only 10 individuals, of whom 3 were females. No differences in fat oxidation were observed in the other populations.

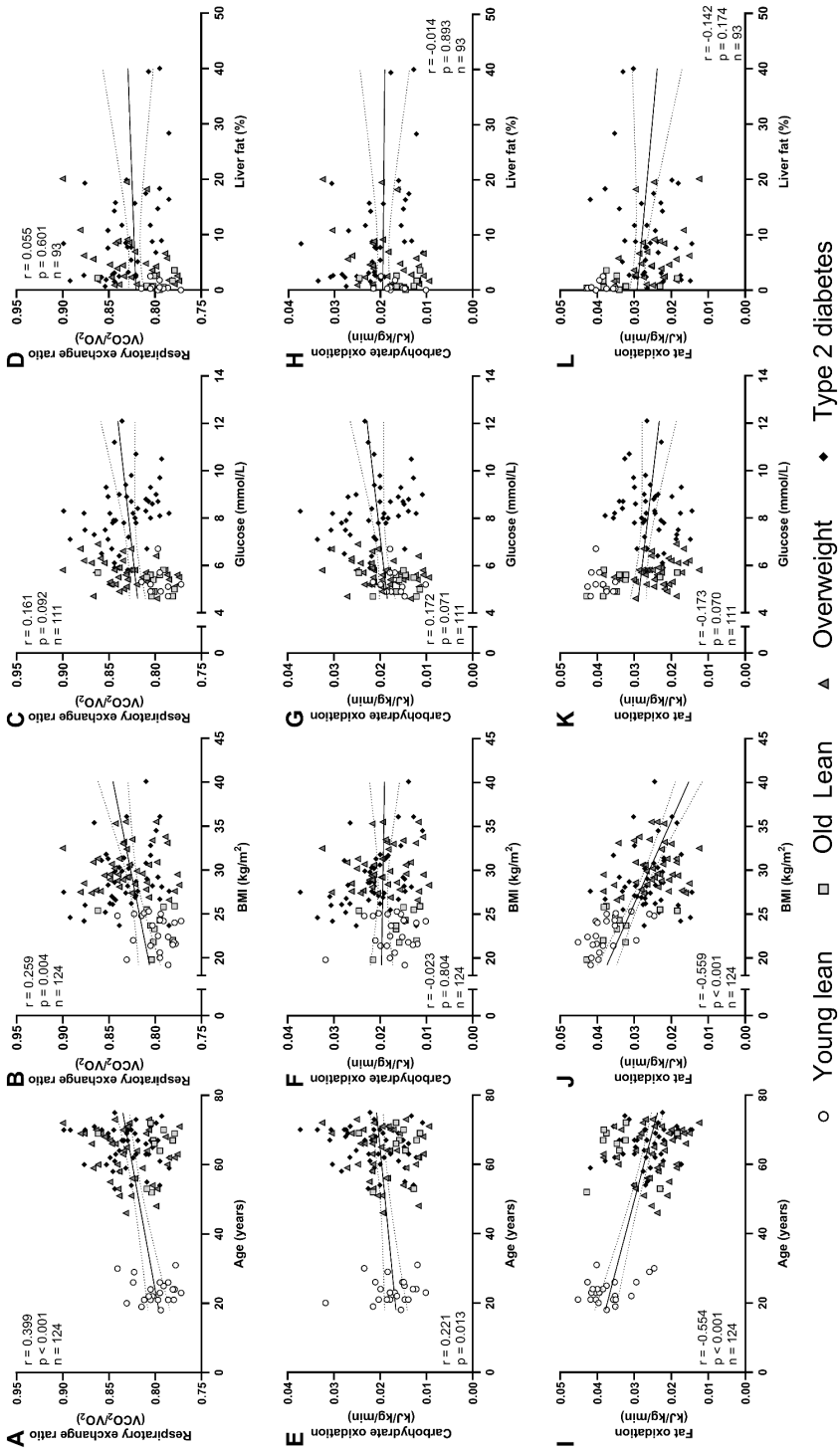
A
Respiratory exchange ratio (VCO2/VO2)
r = 0.399
p < 0.001
n = 124
Age (years)
B
Respiratory exchange ratio (VCO2/VO2)
r = 0.259
p = 0.004
n = 124
BMI (kg/m2)
C
Respiratory exchange ratio (VCO2/VO2)
r = 0.161
p = 0.092
n = 111
Glucose (mmol/L)
D
Respiratory exchange ratio (VCO2/VO2)
r = 0.055
p = 0.601
n = 93
Liver fat (%)
E
Carbohydrate oxidation (kJ/kg/min)
r = 0.221
p = 0.013
Age (years)
F
Carbohydrate oxidation (kJ/kg/min)
r = -0.023
p = 0.804
n = 124
BMI (kg/m2)
G
Carbohydrate oxidation (kJ/kg/min)
r = 0.172
p = 0.071
n = 111
Glucose (mmol/L)
H
Carbohydrate oxidation (kJ/kg/min)
r = -0.014
p = 0.893
n = 93
Liver fat (%)
I
Fat oxidation (kJ/kg/min)
r = -0.554
p < 0.001
n = 124
Age (years)
J
Fat oxidation (kJ/kg/min)
r = -0.559
p < 0.001
n = 124
BMI (kg/m2)
K
Fat oxidation (kJ/kg/min)
r = -0.173
p = 0.070
n = 111
Glucose (mmol/L)
L
Fat oxidation (kJ/kg/min)
r = -0.142
p = 0.174
n = 93
Liver fat (%)
Young lean
Old Lean
Overweight
Type 2 diabetes

Figure 2: Bivariate Pearson correlation coefficients between the respiratory exchange ratio, carbohydrate oxidation, fat oxidation, and baseline characteristics. A-D: Correlation between nocturnal respiratory exchange ratio and age (**A**), BMI (**B**), fasting glucose (**C**), and liver fat percentage (**D**); **E - H**: Correlation between nocturnal carbohydrate oxidation and age (**E**), BMI (**F**), fasting glucose (**G**), and liver fat percentage (**H**); **I - L**: Correlation between nocturnal fat oxidation and age (**I**), BMI (**J**), fasting glucose (**K**), and liver fat percentage (**L**). Young lean population, n = 23; old lean population, n = 10; overweight population, n = 44; type 2 diabetes population, n=47. Best-fit trend line and 95% confidence intervals are included.

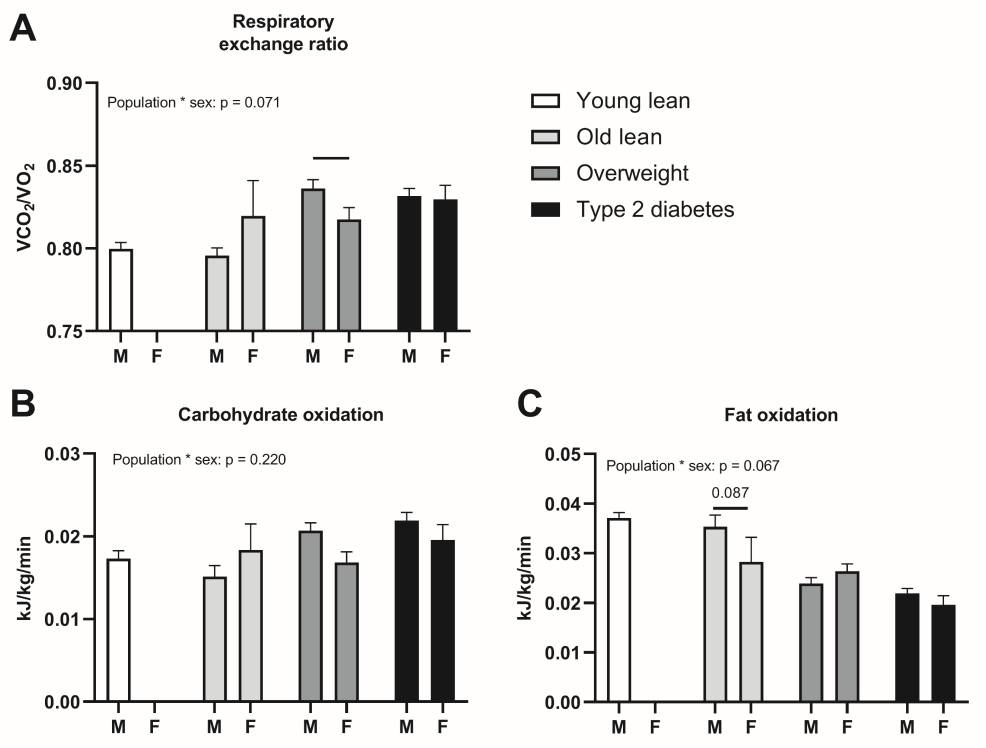

Figure 3: Respiratory exchange ratio and substrate oxidation during the night in different populations split into males and females. A: Respiratory exchange ratio; **B**: Carbohydrate oxidation; **C**: Fat oxidation. For the young lean population, males, n = 23, females n = 0; for the old lean population, males, n = 7, females n = 3; for the overweight population, males, n = 29, females n = 15; for the type 2 diabetes population, males, n = 34, females n = 13. Data are presented as mean ± SEM. Lines indicate significant differences between populations. F, female; M, male.

DISCUSSION

Switching from high carbohydrate oxidation rates during the day to higher fat oxidation rates during the night is a characteristic of having good metabolic health (1). However, we recently showed that metabolically compromised individuals have higher nocturnal carbohydrate oxidation and lower fat oxidation compared to young healthy lean individuals (2, 3). Since the two populations had very different characteristics, with the metabolically compromised individuals being older, being overweight or obese, and having higher fasting plasma glucose levels, the question arises which characteristics are responsible for this lower nocturnal fat oxidation. Here, we combined data from 10 human clinical trials and attempted to unravel the characteristics responsible for nocturnal substrate oxidation. First, we found that nocturnal substrate oxidation is different among the different populations, with higher carbohydrate oxidation and lower fat oxidation rates in the overweight and type 2 diabetes population compared to the young and old lean populations. Interestingly, the old lean population did match their substrate oxidation to the levels of the young lean population, suggesting that age is not a major factor in determining nocturnal substrate oxidation. BMI seems the major factor in nocturnal fat oxidation, with no major contribution of liver fat percentage, and a small contribution of plasma glucose levels.

One of the findings we observed was high levels of nocturnal carbohydrate oxidation and low levels of fat oxidation in the type 2 diabetes population compared to the young lean and old lean populations. Furthermore, the carbohydrate oxidation rate in the overweight population is raising during the night towards the levels observed in the type 2 diabetes population. Since the last meal was ingested around 6 pm, similar to the young lean population, it is unlikely that the dinner provides the glucose for carbohydrate oxidation. This raises the question of where the glucose to fuel the carbohydrate oxidation derives from. Individuals with overweight or obesity, as well as patients with type 2 diabetes, are characterized by higher rates of endogenous glucose production after an overnight fast (15-17), possibly providing the glucose needed for carbohydrate oxidation. This increase in endogenous glucose production is driven by both increased glycogenolysis, as well as increased gluconeogenesis (15). Interestingly, while the rate of endogenous glucose production is generally decreasing during the night (15), we observed higher carbohydrate oxidation rates in the middle and last part of the night. Patients with type 2 diabetes are also characterized by adipose tissue insulin resistance, resulting in increased lipolysis and thereby providing glycerol as a possible substrate for gluconeogenesis (18, 19). Although glycerol normally only contributes for a small part to gluconeogenesis, the contribution of glycerol as a source for gluconeogenesis is increased in patients with type 2 diabetes (19). These findings may suggest that excessive substrate supply in overweight and type 2 diabetes patients may drive gluconeogenesis, which is subsequently attributed to higher levels of carbohydrate oxidation.

It has been shown that individuals with NAFLD are also characterized by higher rates of gluconeogenesis in the overnight fasted state (20, 21). In our studies, many individuals in the overweight or type 2 diabetes populations were characterized with NALFD, which could indicate that NAFLD is associated with high nocturnal carbohydrate oxidation. However, whether NAFLD itself contributes directly to the high carbohydrate oxidation is still unclear, as one study did find higher overnight fasted carbohydrate oxidation rates in patients with NAFLD compared to lean controls (22), while another did not observe any differences in RER between individuals with or without NAFLD (21). Here, we investigate if liver fat percentage was a determinant of nocturnal substrate oxidation, but in line with the observations of Fletcher *et al.* (21), we did not observe an association between nocturnal substrate oxidation and liver fat percentage.

An interesting finding in our study is that the old lean population had a nocturnal RER comparable to the young lean population. This suggests that age is not the driving factor behind the higher nocturnal RER that is observed in the older overweight and/or type 2 diabetes population. Ageing is associated with several metabolic alterations, e.g., decreased mitochondrial function (23), decreased glucose homeostasis (24), and an increase in body weight and redistribution of adipose tissue depots (25). Our analysis suggests that specific body weight, or metabolic alterations associated with overweight and obesity, might be important in the higher RER observed in our older overweight and type 2 diabetes populations. In that respect, one of the strongest correlations we observed was between BMI and nocturnal fat oxidation, with a higher BMI being associated with lower nocturnal fat oxidation. Why being overweight or obese leads to a lower nocturnal fat oxidation cannot be deduced from the current study, as being overweight is associated with different metabolic alterations, e.g., mitochondrial dysfunction (26) and insulin resistance (27). It should be acknowledged that most of our overweight participants are insulin resistant, and it is known that insulin resistance is associated with poor metabolic flexibility (28, 29). Our findings are in line with previous studies reporting an impaired fat oxidation in individuals with obesity in the overnight fasted state (4, 7), although we now showed for the first time that BMI is also associated with nocturnal substrate oxidation.

Although our results give an insight into possible predictors for nocturnal RER and substrate oxidation, the results should be interpreted with some caution. Due to the nature of the human clinical trials conducted, the number of individuals per population in this combined data analysis is not evenly distributed. The young healthy population contains 23 individuals, and the old healthy only 10, whereas the overweight and type 2 diabetes populations contained more individuals. As a result, there is no continuum in age, and the fraction of individuals with a BMI ≤ 25 is the lowest. Furthermore, only a limited number of baseline characteristics were available to include in the analysis. Possibly, we have missed some important predictors, such as insulin sensitivity and mitochondrial function. Therefore, these analyses provide a first insight into the predictors of nocturnal substrate oxidation, although

it would be useful to repeat these analyses in a larger study population in which individuals are more extensively phenotyped.

In conclusion, overweight individuals, as well as patients with type 2 diabetes, have lower nocturnal fat oxidation compared to young and old lean individuals, whereas in type 2 diabetes patients nocturnal carbohydrate oxidation is also increased. Age does not seem to be the major determinant of a high RER observed in the older overweight populations. BMI correlated the strongest with nocturnal fat oxidation, with higher BMI being associated with lower nocturnal fat oxidation. Further studies will need to investigate whether BMI itself or BMI-associated metabolic alterations are responsible for the altered substrate oxidation observed in overweight and obese individuals with and without type 2 diabetes.

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CHAPTER 7

General discussion

Over the past decades, the prevalence of overweight and obesity has increased drastically, and obesity is associated with other chronic metabolic diseases, including cardiovascular disease, non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes. An important hallmark in the development of such chronic metabolic disease is the blunted switch in 24-hour substrate metabolism. The typical switch between predominantly carbohydrate oxidation in the postprandial state and predominantly fat oxidation in the fasted state is blunted, leading to blunted fluctuations in carbohydrate and fat oxidation over 24 hours. Therefore, this thesis focuses on a better understanding of 24-hour substrate metabolism and investigates whether a more pronounced overnight fast can improve metabolic health. Specifically, we investigate whether pharmacological agents and lifestyle interventions can elicit a more pronounced overnight fasted state with an enhanced depletion of hepatic glycogen stores.

How is metabolic flexibility related to 24-hour substrate metabolism?

Metabolic flexibility is originally defined by Kelley *et al.* as the capacity to switch from predominantly fat oxidation and high rates of fatty acid uptake into skeletal muscle during fasting conditions to the suppression of fat oxidation and increased carbohydrate uptake, oxidation, and storage under insulin-stimulated conditions (1). In the following years, it has been observed that insulin-stimulated metabolic flexibility is decreased in various populations, including individuals with overweight or obesity (2, 3), metabolically compromised individuals without type 2 diabetes (4, 5), individuals with NAFLD (6), and in individuals with type 2 diabetes (7). However, substrate oxidation does not only change in response to acute insulin stimulation but is also influenced by eating and fasting and therefore follows a strong day-night rhythm in young lean individuals. Indeed, carbohydrate oxidation is highest and fat oxidation lowest during the day, with highest/lowest values measured in the evening. Carbohydrate oxidation decreased and fat oxidation increased during the night in the fasted state (8). Interestingly, this day-night rhythm in substrate oxidation is blunted in metabolically compromised individuals (individuals with prediabetes), resulting in high rates of carbohydrate oxidation and low rates of fat oxidation during the night (9), which is consistent with the previous reports on metabolic inflexibility in individuals with prediabetes (4, 5). In **chapter 4** we investigated nocturnal substrate oxidation in individuals with NAFLD and observed that they are also characterized by high nocturnal carbohydrate oxidation compared to age-matched lean individuals without NAFLD.

As mentioned above, metabolically compromised individuals with prediabetes, as well as individuals with NAFLD, are characterized by high nocturnal carbohydrate oxidation and low nocturnal fat oxidation, when compared to young, healthy lean individuals (8, 9). Although it is tempting to speculate that this blunting of the eating to fasting transition in substrate oxidation is due to the prediabetic or insulin-resistant state, it can also be due to age, BMI,

liver steatosis, or other characteristics. Therefore, in **chapter 6** we conducted an analysis including a larger number of studies to investigate which characteristics are responsible for the differences observed in nocturnal substrate oxidation. Interestingly, we observed that individuals with overweight or obesity, as well as individuals with type 2 diabetes, have higher nocturnal carbohydrate oxidation rates and lower nocturnal fat oxidation rates compared to age-matched lean individuals. Furthermore, older lean individuals had carbohydrate- and fat oxidation rates similar to those observed in young lean individuals, suggesting that age per se is not the underlying factor. Surprisingly, although we observed in **chapter 4** that individuals with NAFLD were also characterized by high nocturnal carbohydrate oxidation rates and low fat oxidation rates, liver fat percentage per se was not associated with deviations in nocturnal substrate oxidation. On the other hand, higher BMI was associated with changes in substrate oxidation, especially with lower nocturnal fat oxidation rates. It can be questioned whether BMI itself is underlying the impairments in nocturnal fat oxidation, or whether metabolic alterations associated with overweight and obesity are responsible for the lack of switch in substrate oxidation.

As explained in **chapter 2**, obesity is strongly associated with type 2 diabetes, a condition characterized by hyperglycaemia due to impaired insulin-stimulated glucose uptake and elevated endogenous glucose production (EGP). Since hyperglycaemia is known to stimulate carbohydrate oxidation (10), this subsequently results in lower fat oxidation. Indeed, in **chapter 6** we also observed that higher fasting glucose levels tended to associate with higher carbohydrate oxidation and lower fat oxidation. An alternative explanation could be that lower mitochondrial function underly the impaired nocturnal fat oxidation. Thus, mitochondria are key organelles involved in substrate metabolism and an adequate mitochondrial function is essential for substrate oxidation. Obesity, as well as insulin resistance, have been associated with reduced mitochondrial function, including reductions in mitochondrial number and size (11, 12), lower mitochondrial respiration (12, 13), and lower gene expression of proteins involved in oxidative phosphorylation (14). Also, the expression of the fusion protein mitofusin 2 has been shown to be reduced in individuals with obesity and type 2 diabetes (15, 16), possibly leading to a more fragmented mitochondrial network. Therefore, the decreased nocturnal fat oxidation could be due to an overall limited capacity for fat oxidation, caused by mitochondrial dysfunction. However, future studies are needed to test this hypothesis.

Eating-fasting cycle in substrate oxidation

In the normal, healthy condition, during the overnight fasted state, the liver is a crucial organ to maintain plasma glucose levels by upregulating EGP, which provides glucose via the processes of glycogenolysis and gluconeogenesis. Indeed, hepatic glycogen content changes rhythmically over the day, with decreasing levels upon fasting levels, and replenishment

during the postprandial state (17-20). This rhythmicity in glycogen content illustrates that glycogen is mobilized to fuel EGP. It has been shown that EGP is increased in individuals with type 2 diabetes, as well as in overweight or obese individuals without type 2 diabetes (17, 21, 22). However, individuals with type 2 diabetes are also characterized by blunted glycogen breakdown rates (17, 23), suggesting that mainly gluconeogenesis is upregulated in these individuals. This is interesting as it has been shown that depletion of glycogen stores can stimulate fat oxidation (24, 25). Depletion of the hepatic glycogen stores could enhance lipolysis in the white adipose tissue, thereby releasing free fatty acids and glycerol which can be directly oxidized or converted into glucose. The depletion of hepatic glycogen stores can trigger this enhanced lipolysis directly via a liver-brain-adipose neural axis (25), or indirectly via hormones secreted as a result of declining plasma glucose levels. Therefore, it is tempting to speculate that the blunted increase in fat oxidation observed in overweight individuals with and without type 2 diabetes, as observed in **chapter 6**, is due to a blunted decline in hepatic glycogen. To test this hypothesis, in **chapter 4** we aimed to enhance overnight hepatic glycogen depletion by extending the overnight fasting period from 9.5 to 16 hours in individuals with NAFLD and age-matched lean individuals without NAFLD and hypothesised that this would lead to an increase in nocturnal fat oxidation. However, in contrast to our expectations, changes in hepatic glycogen were minor after 16 hours of fasting, and not significantly different from 9.5 hours of fasting, although we observed higher nocturnal fat oxidation after 16 hours of fasting compared to 9.5 hours of fasting. The reason for the lack of decline in glycogen is difficult to deduct from our study but may involve the timing of the meals and the timing of the hepatic glycogen scans.

Other studies investigating the reduction in hepatic glycogen after a (prolonged) overnight fast found reductions of ~19% to ~55% in healthy individuals and ~18% to ~40% in individuals with type 2 diabetes after 10 hours to 17 hours of overnight fasting, respectively (19, 23, 26). Earlier studies also found a pronounced increase in glycogen levels after a meal. Thus, Krssak *et al.* (23) provided a mixed breakfast and lunch at 7 am and 11 am respectively, and a liquid dinner at 5 pm. Hepatic glycogen levels were determined frequently in the postprandial state, as well as the next morning, 14 hours after dinner. Peak glycogen levels were observed 4 to 5 hours after the liquid dinner, and hepatic glycogen levels declined by ~23% in individuals with type 2 diabetes and ~36% in glucose tolerant control individuals from peak levels to 14 hours of fasting. Magnusson *et al.* (26) provided a liquid dinner at 5 pm and measured hepatic glycogen levels 4 hours later when the glycogen peak is expected as well as after a prolonged overnight fast of 17 hours. They found a decrease in glycogen levels of ~40% in individuals with type 2 diabetes and ~55% in age- and BMI-matched control individuals. Macauley *et al.* (19) measured hepatic glycogen at 8 am after an overnight fast of 10 hours. On a separate day, a mixed breakfast, lunch and dinner were provided at 8 am, 12 pm, and 4 pm, respectively, and hepatic glycogen levels were measured 4 hours after dinner. Hepatic glycogen levels declined ~18% and ~19% in individuals with type 2 diabetes and glucose tolerant individuals,

respectively. These studies all show declines in glycogen levels when measured from postprandial peak values. However, in contrast to these studies, we could not measure glycogen levels at peak levels, as we wanted to perform the hepatic glycogen scans at fixed time points to avoid circadian influences. Therefore, we measured hepatic glycogen at 2 pm and 6.30 am the following day, as this comprised the same food intake (evening meal and snack) in both protocols. As a consequence, hepatic glycogen levels were determined 2 hours after lunch, at a moment when 50% of energy intake still needed to be ingested after the first hepatic glycogen scan. These differences in timing may be the reason why we did not observe a decline in glycogen, as is typically observed in other studies. Therefore, further studies examining the effects of prolonging the overnight fast on hepatic glycogen depletion should design a protocol measuring glycogen at peak levels while also avoiding circadian influences.

Can pharmacological agents improve 24-hour and nocturnal substrate metabolism?

As mentioned above, hyperglycaemia is known to increase carbohydrate oxidation and may underly the hampered metabolic changes during the eating-fasting cycle, including the increase in nocturnal fat oxidation that is observed in healthy individuals. In that context, SGLT2 inhibition may be an attractive strategy to restore the typical eating-fasting cycle. SGLT2 inhibitors inhibit the reabsorption of glucose and sodium in the proximal tubule of the kidneys. This results in a glucose excretion of about 60 – 90 grams per day via the urine in individuals with type 2 diabetes. This loss of glucose via the urine results in lower plasma glucose and insulin levels and higher plasma free fatty acids, β-hydroxybutyrate, and glucagon levels (27, 28). Furthermore, fat oxidation increased while carbohydrate oxidation decreased (28-31). However, these effects have all been observed in individuals with type 2 diabetes, and the effects in individuals with prediabetes are yet unknown. Therefore, in **chapter 3** of this thesis, we investigated the effects of 2 weeks of treatment with the SGLT2 inhibitor dapagliflozin on substrate metabolism in individuals with prediabetes. Dapagliflozin treatment resulted in a glucose excretion of approximately 36 grams per day, leading to a negative energy balance. Consistent with our hypothesis, dapagliflozin treatment increased 24-hour and nocturnal fat oxidation rates, while carbohydrate oxidation rates were lower. Interestingly, in line with the higher fat oxidation, we also observed an improved *ex vivo* skeletal muscle mitochondrial fat oxidative capacity. This is an important finding because, as described above, both obesity and insulin resistance are associated with impaired mitochondrial oxidative capacity (12, 13). Whether the effect of dapagliflozin on skeletal muscle mitochondrial fat oxidative capacity is the result of changes in mitochondrial number, fragmentation status, or proteins involved in the oxidative phosphorylation itself is still unclear, although we observed a higher protein content of the oxidative phosphorylation complex III.

As a compensatory effect to the loss of glucose via the urine, SGLT2 inhibitors treatment leads to an increased overnight fasted EGP (28-30, 32, 33), by increasing gluconeogenesis and/or glycogenolysis. As described above, depletion of glycogen stores can stimulate fat oxidation (24, 25), which could explain the increased fat oxidation rates upon SGLT2 inhibition as found in **chapter 3**, and in individuals with type 2 diabetes (28-31). Therefore, it could be speculated that the SGLT2 inhibitor-induced increase in EGP is mainly due to an increase in glycogenolysis and not in gluconeogenesis. However, we found that overnight fasted hepatic glycogen levels were only numerically lower after dapagliflozin treatment but the difference did not reach statistical significance. This may indicate that gluconeogenesis is responsible for a major part of the increase in EGP and that this increase in gluconeogenesis can contribute to both retaining glycogen content and maintaining plasma glucose levels. Indeed, it has been shown in healthy individuals that after an overnight fast, one-third of the glucose produced during gluconeogenesis is used to replenish glycogen stores, whereas two-thirds is released into the blood to maintain euglycemic glucose levels (34). The exact contribution of the glucose produced by gluconeogenesis to maintain glucose levels and the replenishment of glycogen in individuals with prediabetes and upon dapagliflozin is currently unknown and may need further investigation. However, techniques to measure gluconeogenesis are not optimized, time-consuming and expensive.

Since we did not observe a significant decline in hepatic glycogen, it is unlikely that the observed increase in fat oxidation upon SGLT2 inhibitor treatment is stimulated by the depletion of the hepatic glycogen stores. Therefore, it is tempting to speculate that urinary glucose excretion is directly responsible for the higher fat oxidation and improved mitochondrial oxidative capacity as there is less glucose available for oxidation. The loss of urinary glucose results in a mild form of caloric restriction, and it has been shown that caloric restriction can improve mitochondrial function (35). Therefore, most likely SGLT2 inhibitor treatment induced caloric restriction-like effects and was thereby able to restore the typical eating-fasting cycle by stimulating fat oxidation, especially during the night.

Can lifestyle interventions improve 24-hour and nocturnal substrate metabolism?

Lifestyle interventions may be an alternative to pharmacological treatment to improve 24-hour substrate metabolism. In our current 24-hour society with continuous food availability, most people tend to spread their food intake over a minimum of 14 hours (36). By spreading food intake over a longer period, the fasting time becomes shorter, preventing a true nocturnal fasting state. Therefore, limiting the time of food intake to daytime only may restore the eating-fasting cycle, as this automatically prolongs the fasting time. Time-restricted eating is gaining more attention, and time-restricted eating lowered body weight (37-40), lowered plasma glucose levels (41, 42), increased fat oxidation (43), and improved insulin sensitivity (44). However, in most of the studies, the eating time window was very

short (less than 8 hours), making it difficult to adhere to the eating times in daily life. Furthermore, the effects of time-restricted eating in individuals with type 2 diabetes are largely unknown. Therefore, in **chapter 5** of this thesis, we investigated the effects of time-restricted eating within a time window of 10 hours on substrate metabolism in individuals with type 2 diabetes. In contrast to our hypothesis, 10-hour time-restricted eating did not alter 24-hour fat oxidation, but 24-hour carbohydrate oxidation was lower. The lower carbohydrate oxidation may be due to lower mean 24-hour glucose levels and less time spent in the high glucose range since hyperglycaemia stimulates carbohydrate oxidation. Interestingly, while increased 24-hour fat oxidation has been observed in another study (43), we did not observe altered fat oxidation. Probably, the fasting time and timing of the last meal could explain these differences, as in the study of Ravussin *et al.* (43) a 6-hour eating window with the last meal at 3 pm was used, while we used a 10-hour eating window with the last meal at 6 pm. This could indicate that a 10-hour eating window with the last meal at 6 pm is not sufficient to induce a true nocturnal fasting state with increased fat oxidation. We also observed that protein oxidation was numerical, although not statistically, higher after 3 weeks of time-restricted eating. This higher protein oxidation has also been observed previously by Ravussin *et al.* (43), and possibly reflects higher amino-acid-driven gluconeogenesis, which could explain why glycogenolysis was not affected by our time-restricted eating protocol. Further studies directly measuring gluconeogenesis are needed to investigate whether gluconeogenesis is increased after prolonging the overnight fast and whether the higher protein oxidation is indeed a reflective of gluconeogenesis.

Concluding remarks and further perspectives

The research described in this thesis aimed to gain a better understanding of 24-hour substrate metabolism and investigated whether a more pronounced overnight fast can improve metabolic health. More specifically, we investigated whether pharmacological agents and lifestyle interventions can elicit a more pronounced overnight fast whith an enhanced depletion of hepatic glycogen. In this thesis, we showed that 24-hour and nocturnal substrate oxidation differs among populations. Furthermore, we found that both SGLT2 inhibitor treatment, as well as prolonged overnight fasting, could elicit a more pronounced overnight fasting state with higher fat oxidation rates, although this seemed not being mediated by depletion of hepatic glycogen stores.

As reviewed in **chapter 2**, type 2 diabetes is a very heterogeneous disease with different underlying causes. Different types of second-line medications have different properties, which can treat the underlying cause of type 2 diabetes. However, already in the prediabetic state, metabolic disturbances are present similar to those observed in type 2 diabetes. Therefore, starting early with treatment might be beneficial. In **chapter 3** we showed that SGLT2 inhibitor treatment in individuals with prediabetes could improve substrate

metabolism, thereby possibly reducing the risk to develop type 2 diabetes. Further studies are needed to examine whether treatment with SGLT2 inhibitors can be safely used in individuals with prediabetes and whether SGLT2 inhibition can indeed prevent the development to type 2 diabetes.

Another finding that needs more investigation is the blunted decline in overnight hepatic glycogen levels in individuals with different metabolic disturbances. Even after prolonged overnight fasting and SGLT2 inhibition induced loss of urinary glucose, the decline in hepatic glycogen was minor. Therefore, it would be interesting to directly measure the contribution of nocturnal glycogenolysis and gluconeogenesis under conditions of extended fasting or calorie restriction. This would provide more detail about the contribution of glycogenolysis and gluconeogenesis to nocturnal glucose homeostasis, and could reveal to what extent gluconeogenesis is used to replenish glycogen stores.

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ADDENDUM

IMPACT PARAGRAPH

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

This thesis aimed to investigate if a more pronounced fasting state during the night can improve metabolic health, with a special focus on substrate metabolism and liver glycogen. Normally, the body switches throughout 24 hours from glucose to fat oxidation and vice versa, with more glucose oxidation during the day and more fat oxidation during the night. This metabolic flexibility allows the body to use the nutrients which are available at the time, for example via meals. In individuals with overweight or obesity, and in individuals with metabolic diseases related to overweight such as non-alcoholic fatty liver disease (NAFLD), and type 2 diabetes, this flexibility in switching in substrate oxidation is diminished. Glycogen is a form of sugar stored in the liver and muscle. During the night, while fasting, glycogen is converted into glucose, which can be used as an energy source for the brain. The decrease of glycogen in the liver is potentially important for metabolic health, as low glycogen stores are known to stimulate the oxidation of fat. In this thesis, we wanted to investigate whether we can improve substrate oxidation by lowering hepatic glycogen levels via a pharmacological and lifestyle approach.

In **chapter 2**, the heterogeneity of type 2 diabetes, and the effects of different medication classes on insulin sensitivity and β-cell function were reviewed. Current treatment strategies are mainly based on lowering glucose levels; however, for an optimal treatment strategy, it is also necessary to treat the underlying cause of type 2 diabetes. From this review, we conclude that the choice of anti-diabetes medication can be better targeted to treat certain underlying causes. Especially medications that lowers body weight, such as SGLT2 inhibitors and GLP1-receptor agonists, should be used more, as obesity is a major underlying cause of type 2 diabetes. This review should raise the awareness in researchers and treating physicians of the heterogeneity within type 2 diabetes patients and how different aspects of diabetes can be addressed by various treatment options.

In **chapter 3** we investigated the effects of the pharmacological agent dapagliflozin, an SGLT2 inhibitor, in individuals with prediabetes. In patients with type 2 diabetes, SGLT2 inhibition has positive effects on blood glucose values and body weight. Because individuals with prediabetes have metabolic disturbances similar to the disturbances observed in patients with type 2 diabetes, SGLT2 inhibitor treatment might also have positive effects in individuals with prediabetes. We showed that treatment with an SGLT2 inhibitor in individuals with prediabetes increased fat oxidation, and improved muscle mitochondrial function. Therefore, we conclude that SGLT2 inhibitor treatment can be an effective treatment strategy in individuals with prediabetes, thereby reducing the risk to develop type 2 diabetes. The results

of this study set the stage for further research, investigating the benefits of SGTLT2 inhibition by treating individuals at an early stage in diabetes development, even before the disease manifests itself clinically.

Although treatment with a pharmacological agent is one way to improve metabolic health, it is also important to see what can be achieved with lifestyle changes. Therefore, in **chapter 4** we investigated if prolonging the overnight fast acutely by 6.5 hours beneficial effects on metabolic health in overweight individuals with NAFLD, and in lean individuals without NAFLD. We also investigated if liver glycogen stores were affected by extending the overnight fasting time. We prolonged the overnight fast by giving the last meal of the day at either 4.30 pm or 11 pm. We showed that prolonging the overnight fast acutely results in lower glucose oxidation and higher fat oxidation during the night. Furthermore, prolonging the overnight fast did not result in lower liver glycogen levels. Therefore, we conclude that acutely prolonging the overnight fast does stimulate fat oxidation, but this is probably not due to a decrease in liver glycogen. Considering the beneficial effects of an early dinner on nocturnal fat oxidation that was found in this study, together with other recent positive metabolic effects of restricting eating time (**chapter 5**), this reinforces current trends of time restricted eating, which can be easily applied in the general population.

In **chapter 5** we investigated if prolonging the overnight fast for 3 weeks has beneficial effects on metabolic health in patients with type 2 diabetes. Participants were instructed to adhere to a time-restricted eating protocol in which they could eat within a 10-hour time window with the latest meal completed no later than 6 pm or a control protocol in which they had to spread their food intake over at least 14 hours. We showed that time-restricted eating for 3 weeks resulted in lower 24-hour glucose oxidation, but no changes in fat oxidation. Furthermore, no differences were observed in liver glycogen. However, time-restricted eating resulted in improved fasting and 24-hours glucose levels. Therefore, we conclude that 3 weeks of time-restricted eating can be an effective treatment strategy to lower plasma glucose levels in patients with type 2 diabetes. This study identifies that time-restricted eating is an efficient and easy to implement lifestyle change that supports the treatment of type 2 diabetes.

Previously, it has been observed that older individuals with prediabetes have lower fat oxidation and higher glucose oxidation during the night compared to young lean individuals. In **chapter 6** we investigated which factor is responsible for this altered substrate oxidation during the night in some individuals. Therefore, we included participants from 10 different studies and divided them into 4 populations. We observed that individuals with overweight with or without type 2 diabetes had the lowest fat oxidation during the night, while overweight individuals with type 2 diabetes had the highest glucose oxidation. Furthermore, we observed that it is unlikely that age is the driving factor for the differences observed. However, BMI may be responsible for the differences observed in fat oxidation. Therefore,

we conclude that BMI might be a major determinant for low fat oxidation during the night. These results advance our understanding of the determinants of substrate oxidation during the night, an emerging field in metabolic research. Therefore, this study can be the basis for other researchers follow-up on this research.

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

The prevalence of overweight and obesity is high and will be increasing in the upcoming years. Together with the rising prevalence of obesity, also the prevalence of diseases associated with obesity, such as NAFLD and type 2 diabetes, will increase. With the knowledge obtained in this thesis, the understanding of the metabolic disturbances observed in individuals with overweight with and without type 2 diabetes has significantly increased.

Further studies can extent on the results in this thesis by investigating the underlying cause for the observed differences in substrate metabolism between individuals, and exploring if medication or lifestyle interventions can stimulate fluctuations in liver glycogen, thereby improving metabolic health in individuals with obesity and related diseases, such as NAFLD and type 2 diabetes. With a better understanding of the underlying mechanisms causing metabolic disturbances in obesity and related diseases, better and more personalized treatments can be developed. As a result, the findings in this thesis may ultimately also contribute to reducing the costs related to health care.

For whom are the results interesting and of relevance?

The results presented in this thesis are of interest to different stakeholders. First, the results are of interest to other researchers working in the field of substrate metabolism, as knowledge on this topic was extended, especially with regard to fluctuations in substrate metabolism over the day and during the night in individuals with different metabolic disturbances. Furthermore, we investigated whether substrate metabolism can be improved by depletion of liver glycogen. With this knowledge, other researchers can develop follow-up studies to further investigate whether the depletion of liver glycogen can alter substrate oxidation during the night, and how such a depletion of glycogen can best be obtained. Ultimately, this knowledge can be used to develop better treatment strategies for the prevention of obesity and type 2 diabetes.

The insights into the role of pharmacological and lifestyle approaches to stimulate substrate metabolism are of interest to medical professionals such as general practitioners, endocrinologists, and dieticians. SGLT2 inhibitors are currently used in the treatment of type 2 diabetes, and to treat individuals with heart failure and chronic kidney disease with and without type 2 diabetes. We here showed that SGLT2 inhibitors have beneficial effects on

metabolism in individuals with prediabetes. Future research, and policy making should discuss if SGLT2 inhibitor treatment might also be used in individuals who are at risk for type 2 diabetes and if it can prevent the development of type 2 diabetes. Furthermore, medical professionals in direct contact with patients with type 2 diabetes can highlight the potential of time-restricted eating to lower blood glucose levels.

Finally, the results in this thesis are also of interest to the general public, especially for those who have obesity, NAFLD, or are at risk for, or diagnosed with type 2 diabetes, as they provide information about metabolic disturbances related to those diseases. This might stimulate individuals to improve their health by changing their lifestyle, for example by following a time-restricted eating protocol.

How can these target groups be involved and informed about the research results, so that the knowledge gained can be used in the future?

The results described in this thesis have been or will be shared with other researchers and medical professionals through publications in international peer-reviewed journals. Furthermore, the data have been or will be presented at national and international conferences. By publishing in journals and presenting at conferences, the results will be available for researchers, medical professionals and all others who are interested. In addition, the results will also be shared on websites, social media and during study participants' events. Participants who took part in the studies were invited to these participant events, in which the study results were presented.

SUMMARY

The prevalence of overweight and obesity is strongly increasing, and also the prevalence of diseases associated with obesity, such as non-alcoholic fatty liver disease (NAFLD), cardiovascular disease, and type 2 diabetes, is increasing. These metabolic diseases are characterised by altered 24-hour metabolism, in which the typical switch between high rates of carbohydrate oxidation in the postprandial state and high rates of fat oxidation in the fasted state is blunted. This leads to less pronounced carbohydrate and fat oxidation fluctuations over 24 hours. The research described in this thesis focuses on a better understanding of 24-hour substrate metabolism and investigates whether interventions that stimulate a more pronounced overnight fast can improve metabolic health.

Type 2 diabetes is a very heterogeneous disease that includes multiple metabolic dysfunctions characterized by hyperglycaemia, which is the result of various degrees of pancreatic β-cell failure and reduced insulin sensitivity. Despite the heterogeneity, there is no further classification of the disease based on the underlying cause of type 2 diabetes. Recently, Ahlqvist and colleagues suggested a new classification system of diabetes, which, at least partly, considers the heterogeneity of type 2 diabetes. In **chapter 2** we reviewed the heterogeneity of type 2 diabetes and discussed the new proposed classification system. Subsequently, we reviewed the effects of different second-line anti-diabetes medication classes on β-cell function, insulin sensitivity, and metabolism, and discussed the future treatment strategies based on the subgroups suggested by Ahlqvist *et al.* From this review, we conclude that current treatment strategies focus primarily on lowering blood glucose and HbA1c levels and on preventing end-organ damage. However, the new classification system gives an insight into the underlying cause of type 2 diabetes, and could therefore provide a basis for a more personalised treatment strategy focused not only on the consequences but also more tailored towards the cause of diabetes in an individual patient.

As mentioned above, one of the disturbances in type 2 diabetes is a disturbed 24h substrate metabolism. Sodium-glucose cotransporter 2 (SGLT2) inhibitor treatment results in the excretion of approximately 60 – 90 grams of glucose per day in patients with type 2 diabetes. It has been reported that SGLT2 inhibitor treatment results in lower 24-hour whole body carbohydrate oxidation and higher fat oxidation, as well as higher diurnal glucagon, free fatty acids, and β-hydroxybutyrate levels, and lower diurnal glucose and insulin levels. Furthermore, endogenous glucose production is higher as a compensatory effect of the loss of glucose via the urine. However, these effects have all been observed in individuals with type 2 diabetes, and the effects in individuals with prediabetes are largely unknown, yet relevant since individuals with prediabetes have metabolic disturbances similar to the disturbances observed in patients with type 2 diabetes. Therefore, in **chapter 3** we

investigated the effects of dapagliflozin, an SGLT2 inhibitor, on substrate metabolism and hepatic glycogen depletion in individuals with prediabetes. We showed that dapagliflozin treatment increased 24-hour and nocturnal fat oxidation and reduced carbohydrate oxidation, without affecting energy expenditure. Interestingly, dapagliflozin treatment did not affect overnight hepatic glycogen depletion, suggesting that predominantly gluconeogenesis is responsible for the increase in endogenous glucose production. Together, we conclude that SGLT2 inhibitor treatment can be an effective treatment strategy to improve metabolic health in individuals with prediabetes.

In addition to investigating the effects of a pharmacological agent on 24h substrate metabolism, lifestyle factors also affect energy and substrate metabolism. In fact, in our 24-hour society, most people tend to spread their food intake over a minimum of 14 hours, thereby decreasing their time to reach a true nocturnal fasting state and thus disturbing substrate metabolism. To investigate whether acutely prolonging the overnight fast would lead to improvements in substrate metabolism and metabolic health, we performed a proof-of-concept study in **chapter 4**. In this study, we investigated whether acutely prolonging the overnight fast by 6.5 hours would lead to more depletion of the hepatic glycogen stores, thereby increasing nocturnal fat oxidation in overweight individuals with NAFLD and in lean individuals without NAFLD. We provided the last meal of the day at either 4.30 pm or 11 pm, leading to an overnight fast of 9.5 hours and 16 hours. We showed that acutely prolonging the overnight fast resulted in higher nocturnal fat oxidation and lower nocturnal carbohydrate oxidation. However, these changes in substrate metabolism were not accompanied by changes in overnight hepatic glycogen depletion. Therefore, we conclude that acutely prolonging the overnight fast does stimulate nocturnal fat oxidation, but this is probably not due to a decrease in liver glycogen.

In **chapter 4**, the acute effects of a prolonged fast were investigated, but it is also important to investigate the longer-term effects of such intervention. Therefore, in **chapter 5**, we investigated the effects of prolonging the overnight fast for 3 weeks on metabolic health in patients with type 2 diabetes. In this study, we investigated whether repeatedly prolonging the overnight fast would lead to more depletion of the hepatic glycogen stores, thereby increasing nocturnal fat oxidation. Participants were instructed to adhere to a time-restricted eating protocol in which they could eat within a 10-hour time window with the latest meal completed no later than 6 pm or a control protocol in which they had to spread their food intake over at least 14 hours. We showed that 3 weeks of prolonging the overnight fasting time lowered 24-hour carbohydrate oxidation, but did not result in higher fat oxidation or changes in hepatic glycogen content. However, fasting and 24-hour glucose levels did improve. We conclude that repeatedly prolonging the overnight fast is effective to lower blood glucose levels, but does not alter fat oxidation or hepatic glycogen stores.

Previously, it has been observed that older individuals with prediabetes have lower nocturnal fat oxidation and higher carbohydrate oxidation when compared to young lean individuals. In **chapter 4 of** this thesis, we also observed differences in nocturnal substrate metabolism in overweight individuals with NAFLD and in age-matched lean individuals without NAFLD. In **chapter 6** we combined the nocturnal substrate oxidation data from participants of 10 different studies and divided the participants into 4 populations depending on several participant characteristics. We observed that overweight individuals with or without type 2 diabetes had the lowest nocturnal fat oxidation, while overweight individuals with type 2 diabetes had the nocturnal highest carbohydrate oxidation. Furthermore, we observed that young and old lean individuals had similar nocturnal fat and carbohydrate oxidation. Next, we found a strong correlation between nocturnal fat oxidation and BMI. Therefore, we conclude that BMI might be a major determinant of low nocturnal fat oxidation.

In this thesis we investigated 24-hour and nocturnal substrate metabolism, specifically focused on whether a more pronounced overnight fast can improve metabolic health. In **chapter 7** we discussed all our findings and conclude that individuals with overweight or obesity have a disturbed 24-hour substrate metabolism and that this can be improved by pharmacological treatment and lifestyle intervention eliciting a more pronounced overnight fast.

SAMENVATTING

De prevalentie van overgewicht en obesitas neemt sterk toe, en ook de prevalentie van ziekten die gepaard gaan met obesitas, zoals niet-alcoholische vette lever ziekte (NAFLD), hart- en vaatziekten en type 2 diabetes, neemt toe. Deze stofwisselingsziekten worden gekenmerkt door een veranderd 24-uurs metabolisme, waarbij de typische omschakeling tussen een hoge koolhydraatoxidatie in de postprandiale toestand en een hoge vetoxidatie in de gevaste toestand wordt onderdrukt. Dit leidt tot minder uitgesproken koolhydraat- en vetoxidatieschommelingen gedurende 24 uur. Het onderzoek beschreven in dit proefschrift richt zich op een beter begrip van het 24-uurs substraatmetabolisme en onderzoekt of interventies die een meer uitgesproken gevaste toestand gedurende de nacht stimuleren de metabole gezondheid kunnen verbeteren.

Type 2 diabetes is een zeer heterogene ziekte die meerdere metabole disfuncties omvat welke worden gekenmerkt door een verhoogde suikerspiegel. Dit is het gevolg is van verschillende gradaties van β-cel falen in de alvleesklier en verminderde insulinegevoeligheid. Ondanks de heterogeniteit bestaat er geen verdere classificatie van de ziekte op basis van de onderliggende oorzaak van type 2 diabetes. Onlangs hebben Ahlqvist en collega's een nieuw classificatiesysteem voor diabetes voorgesteld, waarin in ieder geval gedeeltelijk rekening wordt gehouden met de heterogeniteit van type 2 diabetes. In **hoofdstuk 2** hebben wij de heterogeniteit van type 2 diabetes geëvalueerd en het nieuwe voorgestelde classificatiesysteem besproken. Vervolgens hebben we de effecten van verschillende tweedelijns anti-diabetes medicatieklassen op de β-cel functie, insulinegevoeligheid en metabolisme besproken, en de toekomstige behandelingsstrategieën op basis van de door Ahlqvist en collega's voorgestelde subgroepen besproken. Uit dit overzicht concluderen we dat de huidige behandelingsstrategieën zich primair richten op het verlagen van het bloedglucose en HbA1c en op het voorkomen van schade aan de eindorganen. Het nieuwe classificatiesysteem geeft echter inzicht in de onderliggende oorzaak van type 2 diabetes, en zou daarom een basis kunnen vormen voor een meer gepersonaliseerde behandelingsstrategie die niet alleen gericht is op de gevolgen, maar ook meer toegesneden is op de oorzaak van diabetes bij een individuele patiënt.

Zoals hierboven vermeld, is een van de verstoringen bij type 2 diabetes een verstoord 24-uurs substraatmetabolisme. Behandeling met natrium-glucose-cotransporter 2 (SGLT2) remmers leidt tot de uitscheiding van ongeveer 60-90 gram glucose per dag bij patiënten met type 2 diabetes. Eerder is aangetoond dat de behandeling met SGLT2-remmers leidt tot een lagere 24-uurs koolhydraatoxidatie in het gehele lichaam en een hogere vetoxidatie, alsook tot hogere glucagon-, vrije vetzuren- en β-hydroxybutyraat niveaus, en lagere glucose- en insuline niveaus. Bovendien is de endogene glucoseproductie hoger ter compensatie voor het verlies van glucose via de urine. Deze effecten zijn echter allemaal waargenomen bij mensen met

type 2 diabetes, en de effecten bij mensen met prediabetes zijn grotendeels onbekend. Toch is dit relevant omdat mensen met prediabetes metabole afwijkingen hebben die vergelijkbaar zijn met de afwijkingen die worden waargenomen bij patiënten met type 2 diabetes. Daarom onderzochten we in **hoofdstuk 3** de effecten van dapagliflozine (een SGLT2-remmer) op het substraatmetabolisme en de leverglycogeen uitputting bij mensen met prediabetes. We toonden aan dat behandeling met dapagliflozine de 24-uurs en nachtelijke vetoxidatie verhoogde en de koolhydraatoxidatie verlaagde, zonder het energieverbruik te beïnvloeden. Interessant is dat behandeling met dapagliflozine geen invloed had op de nachtelijke leverglycogeen uitputting, wat suggereert dat voornamelijk gluconeogenese verantwoordelijk is voor de toename in endogene glucose productie. Samenvattend concluderen we dat behandeling met een SGLT2-remmer een effectieve behandelingsstrategie kan zijn om de metabole gezondheid te verbeteren bij personen met prediabetes.

Naast farmacologische middelen beïnvloeden ook leefstijlfactoren het energie- en substraatmetabolisme. In onze 24-uurs maatschappij zijn de meeste mensen geneigd hun voedselinname te spreiden over minimaal 14 uur, waardoor ze minder tijd hebben om een ware staat van nachtelijk vasten te bereiken en dus het substraatmetabolisme verstoren. Om te onderzoeken of het acuut verlengen van het vasten gedurende de nacht zou leiden tot verbeteringen in het substraatmetabolisme en de metabole gezondheid, hebben we in **hoofdstuk 4** een 'proof-of-concept' studie uitgevoerd. In deze studie hebben we onderzocht of het acuut verlengen van het nachtelijke vasten met 6,5 uur zou leiden tot een grotere uitputting van de leverglycogeen voorraden, waardoor de nachtelijke vetoxidatie zou toenemen bij personen met overgewicht met NAFLD en bij slanke personen zonder NAFLD. Wij gaven de laatste maaltijd van de dag om 16.30 uur of om 23.00 uur, wat leidde tot een gevaste tijd van 9,5 uur of 16 uur. Wij hebben aangetoond dat het acuut verlengen van het nachtelijk vasten resulteerde in een hogere nachtelijke vetoxidatie en een lagere nachtelijke koolhydraatoxidatie. Deze veranderingen in het substraatmetabolisme gingen echter niet gepaard met veranderingen in de leverglycogeen uitputting gedurende de nacht. Daarom concluderen wij dat een acute verlenging van de nachtelijke gevaste toestand de nachtelijke vetoxidatie stimuleert, maar dat dit waarschijnlijk niet te wijten is aan een afname van het leverglycogeen.

In **hoofdstuk 4** werden de acute effecten van het acuut verlengen van het nachtelijk vasten onderzocht, maar het is ook belangrijk om de effecten van een dergelijke interventie op de langere termijn te onderzoeken. Daarom hebben we in **hoofdstuk 5** onderzocht wat de effecten zijn van langdurig vasten gedurende 3 weken op de metabole gezondheid bij patiënten met type 2 diabetes. In deze studie werd onderzocht of het herhaaldelijk langdurig vasten zou leiden tot een grotere uitputting van de leverglycogeen voorraden, waardoor de nachtelijke vetoxidatie zou toenemen. De deelnemers kregen de instructie om zich te houden aan een 'time-restricted eating' protocol waarbij ze binnen een tijdsbestek van 10 uur

mochten eten en de laatste maaltijd niet later dan 18.00 uur mochten nuttigen, of aan een controleprotocol waarbij ze hun voedselinname over ten minste 14 uur moesten spreiden. Wij toonden aan dat 3 weken langdurig vasten de 24-uurs koolhydraatoxidatie verlaagde, maar niet resulteerde in een hogere vetoxidatie of veranderingen in de leverglycogeen voorraden. De nuchtere en 24-uurs glucosespiegels verbeterden echter wel. Wij concluderen dat herhaaldelijk langdurig vasten effectief is om de glucosespiegels te verlagen, maar geen verandering teweegbrengt in de vetoxidatie of de leverglycogeen voorraden.

Eerder is waargenomen dat oudere mensen met prediabetes een lagere nachtelijke vetoxidatie hebben en een hogere koolhydraatoxidatie in vergelijking met jonge, magere mensen. In **hoofdstuk 4** van dit proefschrift hebben we ook verschillen waargenomen in het nachtelijke substraat metabolisme bij personen met overgewicht met NAFLD en bij leeftijdsgenoten zonder NAFLD. In **hoofdstuk 6** combineerden we de nachtelijke substraat oxidatie data van deelnemers van 10 verschillende studies en verdeelden de deelnemers in 4 populaties, afhankelijk van verschillende kenmerken van de deelnemers. We hebben waargenomen dat personen met overgewicht met of zonder type 2 diabetes de laagste nachtelijke vetoxidatie hadden, terwijl personen met overgewicht en met type 2 diabetes de hoogste nachtelijke koolhydraatoxidatie hadden. Verder stelden we vast dat jonge en oude slanke personen een vergelijkbare nachtelijke vet- en koolhydraatoxidatie hadden. Vervolgens vonden we een sterke correlatie tussen nachtelijke vetoxidatie en BMI. Daarom concluderen we dat BMI een belangrijke determinant zou kunnen zijn van lage nachtelijke vetoxidatie.

In dit proefschrift hebben we het 24-uurs en nachtelijke substraatmetabolisme onderzocht, waarbij we ons specifiek hebben gericht op de vraag of een meer uitgesproken gevaste toestand gedurende de nacht de metabole gezondheid kan verbeteren. In **hoofdstuk 7** hebben we al onze bevindingen besproken en geconcludeerd dat individuen met overgewicht of obesitas een verstoord 24-uurs substraatmetabolisme hebben en dat dit kan worden verbeterd door farmacologische behandeling en leefstijlinterventie die een meer uitgesproken gevaste toestand gedurende de nacht uitlokken.

ABOUT THE AUTHOR

Anna Veelen was born on December 9th, 1994 in Westervoort, the Netherlands. After finishing secondary education at Arentheem College Arnhem in 2013, she started the bachelor Biomedical Sciences at Maastricht University. Within the bachelor she specialised in the direction of Biological Health Sciences.

After successfully completing her Bachelor's degree in 2016, Anna continued her education with the Master Biomedical Sciences at Maastricht University. As part of her master she performed internships at the department of Internal Medicine and at the department of Nutrition and Movement Sciences, studying the effect of insulin on the macrocirculation, and investigating insulin sensitivity and metabolic flexibility in individuals with and without type 2 diabetes. In 2018 Anna received her Master's degree in Biomedical Sciences, with a specialization in Nutrition and Metabolism.

In 2018, Anna started her PhD at Maastricht University at the department of Nutrition and Movement Sciences under supervision of prof. dr. Patrick Schrauwen, prof. dr. Vera Schrauwen-Hinderling, and dr. Esther Phielix. The research conducted during this period, as described in this PhD thesis, encompasses human studies focused on improving flexibility in substrate metabolism via pharmacological and lifestyle approaches.

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A. Veelen*, E. Erazo Tapia*, J. Oscarsson, P. Schrauwen. Type 2 diabetes subgroups and potential medication strategies in relation to effects on insulin resistance and beta-cell function: A step toward personalized diabetes treatment?
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In preparation

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Lieve **Anny**, ik geniet nog altijd van de bijzondere band die wij hebben. Al vanaf mijn geboorte ben je een heel groot onderdeel van mijn leven. De eerste twaalf jaar als vaste oppas, maar wat ben ik blij dat nog steeds contact hebben. Ook al zien wij elkaar nu niet meer zo vaak, ik geniet elke keer weer van de momenten waarop wij samen een kopje thee drinken en bij kletsen.

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