Universidade de Lisboa
Faculdade de Motricidade Humana

The Ex-Timing Project: Evaluating morning, afternoon, and evening exercise on the circadian clock in individuals with type 2 diabetes — preliminary results of a randomized crossover study protocol.

Dissertação elaborada com vista à obtenção do Grau de Mestre em

Exercício e Saúde

Orientador: Professor Doutor João Pedro Reis Magalhães

PRESIDENTE

Doutor Rúben Samuel Cordeiro Francisco, assistente convidado da Faculdade de Motricidade Humana da Universidade de Lisboa.

VOGAIS

Doutor João Pedro Reis Magalhães, professor auxiliar da Faculdade de Motricidade Humana da Universidade de Lisboa.

Doutora Sónia Isabel do Vale Fernandes, professora auxiliar da Faculdade de Medicina da Universidade de Lisboa.

André Tomás Vieira

2025

Acknowledgments/Agradecimentos

A paixão pelo desporto e pelo exercício sempre esteve presente na minha vida e foi isso que me fez decidir que era isto que queria para a minha vida. Nunca soube bem o que iria fazer, mas sabia que queria estudar Ciências do Desporto. A licenciatura fez-me perceber que não era o futebol, nem as aulas de grupo que me interessavam, mas sim a investigação e procura pelo conhecimento. Segui para o mestrado de Exercício e Saúde ainda na dúvida de o que seria o meu futuro, mas logo no início do ano falaram-me de um projeto com pessoas com diabetes tipo 2, “acho que é o Ex-Timing mas ainda não começou as recolhas”. Ao que parece foi o projeto que fez crescer o gosto pela investigação dentro de mim. Abracei este projeto como se fosse meu, nem sempre foi fácil, mas foi este projeto que me fez conhecer muitas pessoas, desde colegas e professores que se tornaram amigos, a participantes com quem posso contar para além fora do contexto académico. E desta forma, quero agradecer a todos os participantes, sem exceção, pelas conversas que tínhamos durante os treinos, pelos conhecimentos que me transmitiam sobre os mais diversos temas, pela boa disposição com que vinham aos treinos e pelo compromisso e seriedade com que encararam este desafio. Devo muita da minha experiência enquanto mestrando a todos estes participantes sem eles não se fazem os projetos de investigação. Um grande obrigado a todos!!

Passando agora a quem me acompanhou todos os dias, em primeiro lugar, Professor João, quero agradecer pela forma como leciona as aulas de exercício na saúde e na doença, foi aí que realmente percebi que era isto que realmente me interessava. Quero também agradecer todas as oportunidades que me deu, todos os desafios que me lançou e todas as vezes me relembrou que era Eu-EXTIMING-Faculdade-Mundo e que tudo naqueles momentos dependia da minha dedicação ao projeto. Obrigado, também por toda a ajuda que me deu ao longo de todo o mestrado e por todas a vezes que perguntava como estava a moral, sempre pronto a levantá-la com um discurso motivacional. À Estela um grande obrigado, foi contigo que comecei as recolhas do EX-TIMING, completamente lançados aos leões, mas no fim foi isso que nos fez estar preparados para resolver os problemas que foram surgindo. Ao Natã por toda ajuda no EX-TIMING, obrigado por teres também assumido este projeto como teu. Agradeço-te também pelas conversas e cafés no gabinete, eram esses momentos que nos voltavam a dar alento para continuar naqueles dias que começavam bem cedo e acabavam já de noite. Ao restante Laboratório de exercício e saúde, à Ana, à Inês, ao Gil, ao Rúben, e ao Filipe, um grande obrigado por toda a disponibilidade, sempre prontos a ajudar-me e obrigado por todos os concelhos que

me foram dando ao longo deste ano e alguns meses. Por fim, quero deixar também um obrigado ao Martim e à Daniela, pela ajuda com os treinos naqueles momentos de maior aperto.

Aos meus amigos do mestrado, Sofia e Duarte, muito obrigado, o alcançar desta meta também se deve a vocês que me acompanharam em todas as aulas e trabalhos. À sofia pelo seu pensamento critico que me levava sempre a questionar o porquê das coisas. Ao Duarte sempre disposto a trabalhar ao máximo nos nossos trabalhos de grupo e pronto para oferecer uma ida à luz ver o Benfica.

Fora do ambiente académico, em primeiro lugar, quero agradecer aos meus Pais, Pai, Mãe, muito obrigado por acreditarem sempre em mim e nunca me pressionarem a nada. Obrigado por me deixarem sempre fazer o meu percurso sem duvidar de que estava a fazer o caminho certo. À Mana, ao Alexandre e à Matilde, por me aturarem em vossa casa quando estava cansado e não me apetecia fazer mais nada da faculdade, era aí que ignorava facilmente todas as outras responsabilidades. Maria, muito obrigado por toda a paciência que tiveste ao longo deste processo, era contigo que falava das minhas irritações e repetia vezes sem conta as mesmas coisas. Agradeço todas as idas mensais aos restaurantes de que sabem tão bem e todos os momentos que passamos juntos, sem ter de pensar em mais nada. Por último, não posso deixar de agradecer aos meus amigos, por todos os convívios ao fim de semana que serviam para relaxar das semanas longas e intensas na faculdade.

No fundo, agradeço a todos os que se cruzaram comigo durante este processo e um grande obrigado ao EX-TIMING que foi o projeto que me fez crescer muito ao nível pessoal e académico. Vamos ao próximo, com a mesma vontade e dedicação.

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Abbreviations

AMPK - Adenosine monophosphate-activated protein kinase

AP - Afternoon period

ATP - Adenosine triphosphate

AUCtotal - Total area under the curve

DAG - Diacylglycerol

FFA - Free fatty acids

GLP-1 - Glucose-like peptide one

GLUT - Glucose transporter

HIIT - High-intensity interval training

IL - Interleukin

MP - Morning period

PDK - 3-phosphoinositide-dependent protein kinase

PI3K - Phosphoinositide 3-kinase

PIP3 - Phosphatidylinositol (3,4,5)-triphosphate

SGLT-2 - Sodium-Glucose Co-Transporter 2

T2DM - Type 2 diabetes mellitus

TNF-α - Tumor necrosis factor alpha

VO_2peak - Peak oxygen consumption

EP - Evening period

Abstract

Purpose: To investigate the impact of exercise timing on the 24-hour glucose area under the curve (AUC_{total}).

Methods: Nine individuals with T2DM participated in an exercise intervention following a crossover design, incorporating three distinct blocks of training: morning period (MP), afternoon period (AP), and evening period (EP). Participants began their first training block, scheduled for morning, afternoon, or evening, occurring in 2 weeks with 6 supervised exercise sessions. Each exercise block was followed by a 14-day washout period. CGM assessed glycemic control outcomes, whereas body composition, cardiorespiratory fitness, and 24-hour movement were analyzed as possible confounding factors. Linear mixed models were used to model the outcomes.

Results: After adjusting for sex, age, and treatment order, there were no significant between-group changes in CGM for any of the training blocks AUC_{total} (MP vs. AP: β=-2.0; -18.8, 14.7); (MP vs. EP: β=0.04; -16.1,16.2); (EP vs. AP: β=-2.06; -18.6, 14.5) a significant time effect ($p<0.05$) was found for within-group changes in AUC_{total} across all training periods. Pairwise comparisons showed a significant reduction in AUC_{total} from the start to the end of each period: -12.39±5.95 for the MP, -14.41±6.21 for the AP, and -12.35±5.73 for the EP.

Conclusion: Our preliminary findings indicate that HIIT effectively improves glycemic control in individuals with T2DM. However, no significant differences were observed in AUC_{total} levels among the three training blocks (MP, AP, EP).

Keywords: Circadian rhythm; Continuous glucose monitoring; Exercise timing; High-intensity interval training; glycemic control.

Resumo

Objetivo: Investigar o impacto do horário do exercício na área abaixo da curva (AUC_{total}) da glucose ao longo de 24 horas através da utilização de monitorização contínua da glicose (CGM).

Métodos: Nove indivíduos com DMT2 participaram numa intervenção com exercício seguindo um desenho crossover, incorporando três blocos distintos de treino: períodos da manhã (MP), da tarde (AP) e da noite (EP). Os participantes realizaram 2 semanas com 6 sessões supervisionadas. Cada bloco de exercício foi seguido de um período de washout de 14 dias. O CGM avaliou os resultados de controlo glicémico, enquanto a composição corporal, a aptidão cardiorrespiratória e a atividade física ao longo de 24 horas foram analisadas como potenciais fatores de confundimento. Modelos lineares mistos foram utilizados para analisar os resultados.

Resultados: Após ajuste para sexo, idade e ordem do tratamento, não foram encontradas diferenças significativas entre os grupos nos valores de CGM para nenhum dos blocos de treino AUC_{total} (MP vs. AP: β=-2.0; -18.8, 14.7); (MP vs. EP: β=0.04; -16.1, 16.2); (EP vs. AP: β=-2.06; -18.6, 14.5). No entanto, foi encontrado um efeito de tempo significativo ($p<0.05$) na AUC_{total} ao longo de todos os períodos de treino. Comparações emparelhadas mostraram uma redução significativa em AUC_{total} do início para o final de cada período: -12.39±5.95 para o MP, -14.41±6.21 para o AP e -12.35±5.73 para o EP.

Conclusão: Os resultados indicam que o HIIT melhora o controlo glicémico em indivíduos com DMT2. No entanto, não foram observadas diferenças significativas na AUC_{total} entre os três blocos de treino.

Palavras-chave: Ritmo circadiano; Monitorização contínua da glicose; Timing do exercício; Treino intervalado de alta intensidade; Controlo glicémico.

Chapter 1

Theoretical Framework

Thesis overview

The global prevalence of type 2 diabetes mellitus (T2DM) is increasing, posing serious implications for cardiovascular diseases and all-cause mortality (Magliano et al., 2021). Exercise, a key component in the treatment plan for enhancing glycemic control and mitigating the development of additional co-morbidities in T2DM individuals, offers multiple metabolic benefits, including improved glycemic control (Kanaley et al., 2022). However, not all T2DM individuals experience improved glycemic control with a given exercise program due to several sources of inter-individual variability in glycemic response, with exercise dose being one of major factors (Solomon, 2018). Even with the integration of higher intensities into exercise programs, the outcomes concerning glycemic control and insulin sensitivity improvements for individuals with T2DM remain susceptible to these sources of inter-individual variability (Liu et al., 2019; Solomon, 2018). Timing of exercise is another source of inter-individual variability, with some studies suggesting that it may affect glycemic control in people with T2DM (Kanaley et al., 2023; Menek & Budak, 2022; Savikj et al., 2019; Savikj et al., 2022). In an era of precision medicine, it is paramount to fully understand the impact of the timing of the day the exercise is performed while controlling other sources of inter-individual variability. This thesis aims to contribute significantly to the future of diabetes treatment by providing preliminary experimental data from a crossover randomized trial on how exercise can impact glycemic control in T2DM. Before laying down the results in chapter two, we will first dwell on the literature review, starting with epidemiology, following the physiopathology of T2DM that goes deeper into insulin resistance on muscle, liver, and adipose tissue, inflammation, and β-cells dysfunction. Moreover, circadian rhythm and its disruption will also be discussed in this chapter. Diabetes treatment is divided into pharmacological treatment and exercise, where it will be discussed how exercise affects glucose delivery, transport, and metabolism, as well as the effect on lipids, inflammation, and overall insulin sensitivity. The last subject in the theoretical framework is exercise timing and its impact on glycemic control. It focuses on which period of the day is better for glycemic control, exposes the observational and experimental studies conducted so far, and identifies some gaps in the literature. In chapter two, the methodology and preliminary results of a random crossover trial (EX-TIMING) will be presented, focusing on which period of day (morning vs afternoon vs evening) is more effective on glycemic control in people with T2DM (mean glucose, AUC_{total}).

Literature Review

1. Diabetes Overview

1.1. Epidemiology of Diabetes

The global prevalence of diabetes has been experiencing a notable increase throughout the last decades, impacting approximately 10.5% of the population aged 20 to 79 years, which amounts to 537 million adults (Magliano et al., 2021). Out of the different types of diabetes, type 2 diabetes is the most common, representing 90% of all diagnosed cases. These high prevalence values are aggravated by another emerging problem, where most of the people with T2DM remain undiagnosed (44.7%) (Magliano et al., 2021). As expected, it is estimated that this prevalence will continue to increase in 2030 and 2040, reaching 643 million and 783 million, respectively, mainly affecting people over 60 years old (Magliano et al., 2021). Thus, it is with no surprise that T2DM is a public health problem that comes with a significant financial burden, impacting healthcare systems, with total expenses currently surging to 966 billion dollars worldwide and expected to increase to 1.05 trillion dollars in 2045 (Magliano et al., 2021). In Portugal, a similar trend can be observed with an increase of 20.5% from 2009 to 2021, with current prevalences set at 14.1% (1.1 million individuals aged 20 to 79 years) (Sociedade Portuguesa de Diabetologia, 2023). This surge in diabetes cases correlates with a corresponding increase in hospital admissions directly attributed to the condition, occurring at a ratio of 1 to 5, implying that one out of every five hospitalizations is directly or indirectly linked to diabetes (Sociedade Portuguesa de Diabetologia, 2023). Parallel to global trends, diabetes imposes substantial financial burdens on the Portuguese healthcare system, with annual costs ranging between 1,400 to 1,700 million euros, constituting 6-7% of the total healthcare expenditure in Portugal (Sociedade Portuguesa de Diabetologia, 2023).

1.2. Physiopathology

Type 2 diabetes is considered a modern disease caused by diverse risk factors that can influence the severity of insulin resistance and beta-cell dysfunction, including genetic predisposition, age, exposure to obesogenic environments and resulting adiposity, dietary patterns, physical activity levels, sedentary behavior, sleep duration, circadian rhythm disturbances, stress levels, as well as comorbidities or treatments that may contribute to the development of the disease (Bellou et al., 2018). Diabetes can be diagnosed by an oral glucose tolerance test ≥ 200 mg/dl after two hours, fasting plasma glucose ≥ 126 mg/dl, and HA1C ≥ 6.5% (American Diabetes Association, 2023). Apart from these criteria, people tend to demonstrate some symptoms like polyuria, polydipsia, dry mouth sensation, and unexplained weight loss (American Diabetes Association, 2023). This well-established metabolic dysfunction is defined by chronic hyperglycemia due to insulin resistance and β-cell dysfunction (Demir et al., 2021). Chronic exposure to a hyperglycemic state increases the likelihood of developing both macrovascular and microvascular complications, which negatively impacts the quality of life and increases morbidity and mortality in individuals with diabetes. In people with T2DM, the risk of these complications is closely associated with poor glycemic control and is aggravated in those with HbA1c values above 7% and with a higher number of years living with the disease. On the macrovascular level, complications can arise from persisting hyperglycemia, leading to a higher incidence of cardiovascular diseases, including coronary artery disease, stroke, and peripheral artery disease (American Diabetes Association, 2023). Likewise, higher glucose blood values can lead to microvascular complications such as retinopathy, peripheral and autonomic neuropathy, and nephropathy (American Diabetes Association, 2023). The following chapters will detail the insulin resistance and beta cell dysfunction mechanisms.

1.2.1. Insulin resistance

Insulin is a hormone produced in the pancreas, specifically in the pancreatic β-cells, and it is responsible for glucose homeostasis with action in different tissues (Petersen & Shulman, 2018). This hormone enhances glucose transport and glycogen synthesis in the muscle, contributing to storage and usage. In the liver, insulin promotes glycogen synthesis and decreases neoglucogenesis, whereas in the white adipose tissue, it suppresses lipolysis and increases glucose transport and lipogenesis (Petersen & Shulman, 2018).

When insulin binds to its receptor, a series of tyrosine and serine phosphorylation starts activating insulin receptor substrates-1 and -2 (Batista et al., 2021; Boucher et al., 2014). This phosphorylation process allows the creation of docking sites for Phosphoinositide 3-

kinase (PI3K), leading to the formation of phosphatidylinositol (3,4,5)-triphosphate (PIP_3) (Batista et al., 2021; Boucher et al., 2014). Afterward, PIP3 activates 3-phosphoinositide-dependent protein kinase (PDK), which in turn activates AKT and AS160 and unlocks glucose transport to the cell due to GLUT (glucose transporter)-4 translocation (Batista et al., 2021; Boucher et al., 2014). Furthermore, after the connection with the insulin receptor, insulin triggers glycogen synthase 1, hexokinase 2, and other parts of the mitochondrial electron transport chain, enhancing glucose metabolism and glycogen synthesis (Batista et al., 2021). When this cascade fails, all the above mechanisms are compromised, which may increase glucose levels in the bloodstream (Batista et al., 2021). Insulin resistance is responsible for a substantial increase in insulin secretion to regulate glucose blood levels, and if maintained unchecked throughout time, it may contribute to pancreatic β-cells dysfunction and T2DM (Petersen & Shulman, 2018). Insulin resistance can be induced by many different sources, including lipotoxicity, inflammation, hyperglycemia, mitochondrial dysfunction, and endoplasmic reticulum stress (Boucher et al., 2014). The presence of these metabolic risk factors negatively affects the tyrosine phosphorylation of insulin receptor substrate-1 and insulin receptor substrate-2 by increasing the ratio of Serine/threonine kinase activation and, hence, reducing the strength of the insulin cascade signal (Boucher et al., 2014). Given the importance of the liver, muscle, and adipose tissue in optimal glycemic control, we will further discuss how insulin resistance impacts each of these organs in the following subchapters (Petersen & Shulman, 2018).

Insulin resistance on adipose tissue

In typical situations, the subcutaneous adipose tissue stores and releases fatty acids into the bloodstream depending on insulin stimulation after a given meal (Smith & Kahn, 2016). In the post-prandial period, when insulin levels are high, we observe a decrease in the lipolytic activity of the adipose tissue with a concomitant increase in lipogenesis and an increase in glucose uptake and utilization (Tchernof & Després, 2013). However, in an obesogenic environment, where a positive energy intake balance prevails, the adipocytes in the subcutaneous adipose tissue cannot expand, releasing free fatty acids to the bloodstream, which will be stored as ectopic fat (Tchernof & Després, 2013). The accumulation of ectopic fat, as well as visceral and intramuscular adiposity, represents a significant risk for metabolic and cardiovascular disease (Tchernof & Després, 2013). Furthermore, visceral adipose tissue has a higher level of insulin resistance when compared to subcutaneous adipose tissue, thus leading to a more pronounced lipolysis during post-prandial and a higher rate of free fatty acids (FFA) turnover (Laviola et al., 2006). If this positive energy balance is maintained, this will become a vicious cycle of overloading

adipocytes and expanding insulin-resistant visceral adipose tissue adipocytes (Smith & Kahn, 2016). During this process, the size increase of subcutaneous adipose tissue and visceral adipose tissue is not just by the hypertrophy of the adipocytes (increasing the size of the cells) but also by adipocyte hyperplasia (increasing the number of fat cells) (Tchernof & Després, 2013). In turn, this will lead to dysfunction and death by hypoxia adipocytes due to low tissue vascularization (Petersen & Shulman, 2018). Because of the apoptosis of adipocyte cells, the monocyte chemoattractant protein 1 will be responsible for recruiting monocytes into the adipocyte's extracellular environment macrophages, increasing macrophage differentiation (Sartipy & Loskutoff, 2003). Due to the macrophage recruitment, cytokines like tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 beta, and IL-6 are released, inducing low-grade inflammation and impacting adipose tissue insulin sensitivity (Sartipy & Loskutoff, 2003). Cytokines like TNF- α contribute to serine phosphorylation, reducing tyrosine phosphorylation, the key chain of the downstream activation of PI3K and GLUT-4 translocation; it also induces lipolysis, enhancing the increase of FFA in the bloodstream (Boucher et al., 2014).

Insulin resistance in muscle

Muscles are one of the most essential tissues for glucose metabolism, given that they are responsible for 25-30% of insulin-stimulated glucose uptake (Petersen & Shulman, 2018). After insulin binds to its receptor, a series of phosphorylation cascades converge in GLUT-4 translocation and, consequently, the entrance of glucose into the cell (Petersen & Shulman, 2018). Insulin-resistant individuals (e.g., obesity and T2DM) tend to show a deficit in glucose transport due to deficient insulin receptors on the cell membrane and reduction of tyrosine phosphorylation of PI3K and AKT, impeding the migration of GLUT-4 to the membrane (Petersen & Shulman, 2018). Apart from transport issues, glucose storage is also disrupted because there are two insulin-dependent processes such as the transcription of hexokinase II, a glycolytic enzyme responsible for the conversion of glucose to glucose-6-phosphate, and glycogen synthase, an anabolic enzyme accountable for glycogen synthesis (Petersen & Shulman, 2018). If insulin signaling is disrupted, none of these processes occur. Some glucose remains in the bloodstream, and the few entering the cell may not be efficiently phosphorylated into glucose-6-phosphate, impacting energy production and storage(Petersen & Shulman, 2018).

As stated before, hypertrophied adipocytes tend to release FFA, which is uptake by different cells and organs, including myocytes, thus contributing to ectopic fat accumulation in the muscle (Galgani et al., 2008). It is known that lipids in the muscle can be used as a source

of energy when exercising; however, when in excess, it may induce insulin resistance (Brøns & Grunnet, 2017). More recently, attention has been given not only to the amount of lipids in the cell but also to the quality of such lipids during the insulin resistance process (i.e., lipid intermediates) (Brøns & Grunnet, 2017). A significant part of these FFA is stored in lipid droplets in the form of triglycerides; the other part can be stored as **l**ong-chain fatty acyl-CoAs, DAG (diacylglycerol), and ceramides (lipid intermediates**)** that activate the protein kinase-C, resulting in serine phosphorylation of the insulin receptor substrate-1, blocking the rest of the downstream cascade that ends with insulin signaling disruption (Brøns & Grunnet, 2017).

Usually, when fasted, the cells prefer lipids as a first-order energy supply (Muoio, 2014). When in a fed state, the mitochondria oxidize glucose rather than fat, but between meals, the fuel selection of mitochondria is a mix of both (Muoio, 2014). This ability to change substrates depending on the meal is called metabolic flexibility (Muoio, 2014). Due to the excess disposal of fatty acids and glucose, the mitochondria are saturated and dysfunctional and unable to choose between all the available substrates (Brøns & Grunnet, 2017). This substrate competition causes mitochondrial stress and overload, promoting reactive oxygen species production, such as H_2O_2, which impairs insulin signaling by disrupting the function of tyrosine phosphatases. Thus, the production of reactive oxygen species by the mitochondria is crucial in the downstream processes of insulin signaling (Muoio, 2014). Beyond that, the endoplasmic reticulum is another organelle under metabolic stress and is affected by mitochondria-produced reactive oxygen species that change insulin signaling (Petersen et al., 2017). This organelle is responsible for the synthesis of many proteins. When this nutrient overload increases the demand for protein synthesis and lipid processing in the endoplasmic reticulum, unfolded proteins accumulate (Hotamisligil, 2010). Endoplasmic reticulum stress affects insulin resistance primarily by activating the unfolded protein response (Hotamisligil, 2010). The unfolded protein response is triggered by the accumulation of misfolded proteins in the endoplasmic reticulum, activating various signaling pathways, including the c-Jun N-terminal kinase inflammatory pathway. c-Jun N-terminal kinase can impair insulin signaling by Serine phosphorylating insulin receptor substrate 1, which inhibits the downstream insulin signaling cascade (Hotamisligil, 2010).

Insulin resistance on the liver

The liver is at the central regulation of glycemic control and is responsible for 90% of endogenous glucose production (Petersen et al., 2017). Hepatic glucose production

appears in many ways, such as gluconeogenesis, glycolysis, glycogenolysis, and glycogen synthesis, depending on the fed or fasted state (Petersen et al., 2017). When the physiological response is not disrupted in a fasted state, the hepatic glucose production is enhanced to maintain euglycemia; in a fed state, hepatic glucose production decreases by the suppression of gluconeogenesis and glycogenolysis by insulin action to maintain euglycemia (Petersen et al., 2017). In T2DM individuals, hepatic insulin resistance leads to an inability to suppress gluconeogenesis and glycogenolysis in the postprandial phase, exacerbating hyperglycemia (Roden & Shulman, 2019). In a similar fashion to the muscle, the liver is also affected by the excess lipids in the bloodstream derived from the adipose tissue, which leads to hepatic steatosis and, in turn, to nonalcoholic fatty liver (Watt et al., 2019). As mentioned in the previous chapter, the quantity of lipids is not the only factor contributing to insulin resistance, but the quality of lipids is crucial to understanding this phenomenon (Petersen et al., 2017). The accumulation of specific lipid intermediates, such as DAG, significantly influences insulin resistance through the activation protein kinase Cε, which inhibits insulin receptor tyrosine phosphorylation and activates the threonine 1160 phosphorylation, impairing the rest downstream of insulin signaling (Petersen et al., 2017). In these conditions of hepatic lipid accumulation, endoplasmic reticulum stress has the same consequence as in the muscle, being another important cause of insulin resistance, ending in a liver without the capacity to interrupt its glucose-releasing (Hotamisligil, 2010).

Beta cells dysfunction

Pancreatic beta cells are responsible for insulin production and secretion, which plays a significant role in the post-prandial state, promoting glucose uptake by the cells and other insulin mechanisms mentioned before (Dludla et al., 2023). The loss of beta cell function is the primary factor in the development of T2DM, usually preceded by insulin resistance (Eizirik et al., 2020). Beta cell mass can be reduced by approximately 40%, with a reported range of 25–60%, impairing the maintenance of glucose homeostasis (Eizirik et al., 2020). This process often begins with excessive caloric intake, leading to obesity and elevated lipid levels in the bloodstream. The subsequent lipid accumulation near vital organs induces insulin resistance and chronic hyperglycemia (Tchernof & Després, 2013). Pancreatic beta cells increase insulin secretion to compensate for elevated glycemia overload, leading to its strain and consequent failure (Kasuga, 2006). Beyond that, the accumulation of lipids on the Langerhans islets contributes to β-cell dysfunction and reduces the β-cells glucose sensitivity and apoptosis of those cells (Dludla et al., 2023). Concomitantly, inflammation due to dysfunctional adipocytes activates pro-inflammatory cytokines like IL-1β, which inhibit β-cells function (Dludla et al., 2023). Moreover, TNF-α is also associated with T2DM

progression, responsible for β-cells apoptosis (Dludla et al., 2023). Oxidative stress also plays an essential role in β-cells dysfunction by the production of reactive oxygen species, a result of chronic exposure to high levels of glucose and FFA and mitochondrial overload (Gerber & Rutter, 2017).

1.2.2. Circadian Rhythm and Glycemic Control

Biologically, internal clocks regulate cellular processes, but several external factors also influence molecular rhythmicity, including light exposure, meal timing, and exercise (Gabriel & Zierath, 2019; Mohawk et al., 2012; Panda, 2016). Those clocks work autonomically with a transcriptional-translational feedback loop by transcriptional activators CLOCK and BMAL1, with a favorable regulatory expression of Per1 and Per2, Cry1, and Cry2 (Mohawk et al., 2012; Panda, 2016). Activating those proteins represses the transcription of CLOCK and BMAL1 by itself and through the accumulation and interaction with nuclear receptor Rev-Erb (α and β) (Gabriel & Zierath, 2019; Panda, 2016). The master clock is the suprachiasmatic nucleus; it maintains the metabolism during a 24-hour rhythm from activity to rest and feeding to fasting, primarily dependent on light sensed by receptors on the retina and other external factors (Panda, 2016; Poggiogalle et al., 2018). At the periphery, many clocks in the liver, pancreas, skeletal muscle, and adipose tissue combine information from the central clock and environmental factors (Poggiogalle et al., 2018). For example, muscle has a vital role in glucose uptake and metabolism, as far as its clock prepares the muscle tissue to shift from rest/fasting to active/feeding (Schiaffino et al., 2016).

The interaction between the central clock and peripheral clocks defines our circadian rhythm (Poggiogalle et al., 2018), and different factors, such as artificial lights, high-density caloric and mistiming food intake, shift work, social jet lag, and others, can affect the alignment of the circadian rhythm and, consequently, lead to the development of T2DM (Mason et al., 2020). This misalignment can affect the normal metabolism of different substrates in the various organs and response to exercise (Gabriel & Zierath, 2019; Mason et al., 2020). Among several people with T2DM, it is clear that they consume a quarter of their daily energy intake in the late evening time (when glucose tolerance is decreased), which can lead to impaired glucose tolerance for many reasons, such as consuming food during an unfavorable circadian phase, eating when melatonin levels are elevated, delayed meal timing leading to internal misalignment (Mason et al., 2020). Another important issue is related to the dawn phenomenon, a metabolic disturbance related to the unnatural increase in blood glucose levels in the early morning hours, associated with endogenous glucose production arising from the liver (Monnier et al., 2013). This contributes approximately 0.4%

to the HbA1c level and impacts overall glycemic control in individuals with type 2 diabetes (Monnier et al., 2013). The relationship between the dawn phenomenon and circadian disruption is not a recent subject; in the early 90´s, Shapiro and his colleagues noted that it is related to the circadian variation of cortisol (Shapiro et al., 1991). In 2006, it was detected that this increase in glucose is mediated by suprachiasmatic nucleus-induced sympathetic stimulation, resulting in enhanced glycogenolysis in the liver without an increase in insulin (Radziuk & Pye, 2006). It was noticed that people with T2DM sometimes can show a more prolonged dawn phenomenon (extended down phenomenon) (Peng et al., 2022). This prolonged phenomenon is associated with different expressions of Rev-Erb (α and β) at suprachiasmatic nucleus GABA neurons, leading to glucose intolerance and insulin resistance in the liver in the morning (Ding et al., 2021). All this data suggests that glucose metabolism is clearly under circadian influence.

1.3. Diabetes treatment

Despite the proven benefits of medication, evidence highlights that optimal diabetes care extends beyond pharmacological interventions such as lifestyle factors, including structured physical activity, nutrition, and weight management, which play a crucial role in improving both glycemic outcomes and overall quality of life (Davies et al., 2022). Advances in pharmacological treatments have significantly improved glycemic control and mitigated complications, with medications like Glucagon-like peptide-1 (GLP-1) receptor agonists and sodium-glucose co-transporter type 2 (SGLT2) inhibitors offering glucose-lowering benefits and cardiometabolic protection (Davies et al., 2022). These therapies are increasingly tailored to patient profiles, demonstrating efficacy in reducing cardiovascular and kidney-related risks while addressing the broader metabolic spectrum of diabetes (Davies et al., 2022). This chapter explores the benefits of current medications in managing T2DM while emphasizing their integration with lifestyle interventions.

1.3.1. Pharmacological treatment

There are many medical treatments for diabetes, all of which aim to lower glucose levels. This chapter will discuss medications used in this thesis, such as metformin, SGLT2 inhibitors, and GLP-1 analogs.

Metformin

Metformin is one of the most used medications for T2DM treatment because of its effectiveness in glucose-lowering abilities, with effects on HbA1c, weight neutral, and in-expensive medication (American Diabetes Association, 2023). This first-line therapy acts in

the liver by reducing the hepatic glucose production by about 15-20% due to the reduction of hepatic gluconeogenesis (LaMoia & Shulman, 2021). At the muscle level, metformin also affects glucose metabolism by increasing insulin-stimulated glucose uptake (LaMoia & Shulman, 2021).

SGLT2 Inhibitors

Inhibitors of sodium-glucose co-transporter type 2 are one of the pharmacological treatments for T2DM, reducing hyperglycemia with less risk of hypoglycemia occurrence and benefits on weight loss (Scheen, 2015). This type of therapy acts on kidney function, blocking the reabsorption of glucose, resulting in its release into the urine, with a consequent increase in glucosuria (Scheen, 2015).

GLP-1 analogs

Glucagon-like peptide-1 is an incretin that acts in multiple organs. It is produced in the small bowel and colon in response to food intake (Drucker, 2006). GLP-1 acts in different organs through GLP-1 receptors in their membranes, such as pancreatic islets, kidneys, stomach, heart, and central and peripheral nervous systems (Drucker, 2006). GLP-1 stimulates insulin secretion, decreases glucagon, delays stomach emptying, promotes satiety, enhances β-cells proliferation, reduces apoptosis, and enhances insulin sensitivity in muscle (Drucker, 2006). This incretin, at its native state, has a short lifespan in circulation because it is degraded by dipeptidyl peptidase-4 (Drucker, 2006). Looking at its action in different tissues, it is very plausible that GLP-1 has promising potential in T2DM treatment, but it stays too short in circulation (Meier, 2012). Therefore, GLP-1 receptor agonists were developed to help with T2DM treatment, creating a synthesized GLP-1 resistant to dipeptidyl peptidase-4 action, which can stay in circulation for a more extended period (Meier, 2012). This GLP-1 receptor antagonist can be short-action and long-action; the first ones act mainly in a postprandial state, lowering the glucose by the stomach emptying delay, for example, exenatide and lixisenatide (Meier, 2012). The long-action GLP-1 receptor antagonist, such as liraglutide, albiglutide, exenatide, and dulaglutide, acts primarily via insulin secretion stimulation and decrease of glucagon release, and consequent lower glucose levels (Meier, 2012). More recently, semaglutide is a GLP-1 receptor antagonist used for treating metabolic disorders and shows improvements in weight and consequent cardiometabolic risk factors (Bergmann et al., 2023).

1.3.2. Exercise and glycemic control in T2D

People with T2DM usually have all the metabolic processes around glucose disrupted, including its delivery, transport, and metabolism. Moreover, as explored before, insulin sensitivity, lipids metabolism, and inflammation are concerns when discussing people with this disease. These problems can be dealt with medications. However, those processes can respond to lifestyle interventions, such as physical exercise, that effectively reduce macro and microvascular complications associated with diabetes despite being below the recommended target (Rietz et al., 2022). This chapter will examine how exercise influences the various processes involved in glucose metabolism.

Glucose delivery

In the exercise context, the needs for oxygen and energy substrates are higher than in rest; thus, the blood flow must be greater than in rest (hyperemia), with the flow rate changing from 5 L/min to 20 L/min near maximal intensities (Joyner & Casey, 2015). This leads to higher blood volume reaching the muscles and, with this, higher glucose delivery to the peripheral tissues (Joyner & Casey, 2015; Sylow et al., 2017). Several mechanisms can be the genesis vasodilatation increasing glucose uptake through the increase in microvascular recruitment and blood flow (Sylow et al., 2017), such as adenosine triphosphate (ATP), nitric oxide, and prostaglandins (Rosenmeier et al., 2004).

Firstly, ATP can induce vasodilatation when it binds to receptors of purinergic P_{2y} in the vasculature's endothelial cells (Rosenmeier et al., 2004). After binding these receptors, endothelium-derived hyperpolarization factors, prostaglandins, and nitric oxide are released, promoting vasodilatation (Rosenmeier et al., 2004). Furthermore, ATP can reduce the sympathetic vasoconstrictor activity usually augmented in exercise (Rosenmeier et al., 2004). Next, we have increased nitric oxide expression and secretion due to shear stress, which is responsible for vessel dilation and is positively affected by exercise (Green et al., 2017). Shear stress is the force created by blood flow on the endothelial cells. When flow velocity increases, the shear stress also increases, promoting nitric oxide release by the endothelial cells and consequently causing an acute rise in arterial lumen diameter through dilation (Green et al., 2017; Thijssen et al., 2011). In the medium to long term, these exercise improvements (increased arterial function) can evolve into chronic adaptations (improved arterial structure), resulting in enhanced cardiovascular function (Green et al., 2017). In third place, prostaglandins function through autocrine and paracrine signaling by interacting with prostanoid receptors to activate the endothelial enzyme nitric oxide synthase, which leads to the production and release of nitric oxide (Goto et al., 2007; Hristovska et al., 2007).

Glucose transport

Glucose passes into the cell through GLUT-4 transporters that must be translocated to the cell membrane to establish the connection between the bloodstream and the muscle fibers (Sylow et al., 2017). The exercise glucose transport pathway is an insulin-independent pathway for glucose uptake by the cells; thus, it is an alternative to the insulin pathway to reduce glycemia in people with insulin resistance. Different exercise-dependent mechanisms for GLUT-4 translocation have been identified, such as ATP turnover, calcium-sensitive signaling, and mechanical stress (Sylow et al., 2017). ATP is the energy source for muscle contraction, and its utilization contributes to increased AMP/ATP ratio and the activating of adenosine monophosphate-activated protein kinase (AMPK). In turn, AMPK is a muscle energy sensor that phosphorylates AS160 and consequently releases GLUT-4 from the inner cell cytoplasm to promote glucose uptake (Osorio-Fuentealba et al., 2013; Sylow et al., 2017). Increased calmodulin kinase activity also facilitates glucose uptake by promoting GLUT-4 translocation (Sylow et al., 2017). The activation of this enzyme happens because muscle contraction stimulates the increase of intracellular calcium, which directly affects the activity of calmodulin kinase (Sylow et al., 2017). Lastly, the mechanical stress is sensed by integrins on cell membranes, which are transmembrane proteins that function as receptors, help establish the connection between intra and extracellular environments, activating RAC1 and, consequently, GLUT-4 translocation (Sylow et al., 2017).

Glucose metabolism

The most relevant source of energy in muscle is glucose. During prolonged exercise, the demand for glucose uptake from the bloodstream increases as the body's energy needs rise over time (Sylow et al., 2017). Initially, muscles rely on stored glycogen for energy; however, as exercise continues and glycogen stores deplete, muscles increasingly depend on blood glucose uptake (Sylow et al., 2017). Deeper in this physiological mechanism is the hexokinase, which might explain improvements in glucose uptake and metabolism (Sylow et al., 2017). This enzyme is responsible for the phosphorylation of glucose that enters the cell and is stored or used for ATP production (Sylow et al., 2017). In the exercise context, the demand for glucose is increased, as explained before; however, in the early moments of exercise, there is a more significant amount of glucose-6-phosphate, which is a hexokinase inhibitor, decreasing its activity (Sylow et al., 2017). As exercise continues or its intensity increases, the demand for glucose rises, the free glucose in the cell (glucose that has not been phosphorylated by hexokinase) depletes, and hexokinase activity is no longer inhibited (Sylow et al., 2017). Thus, if hexokinase activity is not inhibited due to the increased glucose requirements from exercise, it keeps its glucose phosphorylation

functions and glycolysis when bound to the mitochondria, enhancing glucose metabolism (Sylow et al., 2017).Exercise also induces changes in mitochondria, specifically when done with higher intensities, by activating peroxisome proliferator-activated receptor-coactivator 1α (Bishop et al., 2019). This transcriptional activator facilitates the expression of genes that encode proteins, leading to an increase in mitochondrial biogenesis (Bishop et al., 2019). Additionally, high-intensity exercise induces improvements in mitochondrial function, content, and cristae density, as well as greater efficiency in the oxidation of substrates such as glucose and lipids, supporting metabolic flexibility (Bishop et al., 2019).

Lipids

People with T2DM and obesity usually have impaired capacity to oxidate fatty acids, primarily due to mitochondrial capacity (Fritzen et al., 2020). To counter this, exercise is an effective way to enhance fatty acid oxidation in insulin-resistant people, independently from weight loss (Fritzen et al., 2020). These improvements in fatty acid oxidation can be explained predominantly by alterations in lipid content and lipid and not so clearly by alterations in lipid intermediaries.

In 2001, it was discovered that endurance athletes tend to show a more significant amount of intra-myocellular lipids compared to healthy non-athlete individuals, just like T2DM people (Goodpaster et al., 2001). However, it was called the “athlete paradox” because these intra-myocellular lipids serve has reserves for prolonged physical exercise without comprising metabolic flexibility and insulin sensitivity (Goodpaster et al., 2001). That discovery was the starting point for understanding how exercise affects intra-myocellular lipids dynamics in people with T2DM (Magalhães et al., 2022). Nevertheless, people with T2DM cannot use intra-myocellular lipids as an energy source because of a chronic higher rate of lipolytic activity and FFA circulation in the bloodstream (Bergman & Goodpaster, 2020). Additionally, compared to athletes, individuals with T2DM have larger lipid droplets near the membrane of fast muscle fibers, with fewer mitochondria available to oxidize a greater amount of lipid content (Bergman & Goodpaster, 2020). So, this leads to the conclusion that the effects of exercise on muscle lipids may not stem from changes in the amount of lipids but rather from the characteristics of the lipid droplets (Bergman & Goodpaster, 2020). With long-term exercise, lipid droplets tend to migrate to intermyofibrillar space, where they will be closer to mitochondria, facilitating lipid oxidation and contributing to better insulin sensitivity (Gemmink et al., 2020; Tarnopolsky et al., 2007).

When speaking of lipid intermediates, it is known that they affect insulin sensitivity, for example, polar lipids (LCA-CoA and acylcarnitine) that directly affect AKT phosphorylation,

DAG sn-1,2 that activate protein kinase C, and ceramides that also decreases AKT activity through protein kinase C activation (Magalhães et al., 2022). Different researchers have tried to find if exercise affects those lipid intermediates, but there is no consensus on exercise's acute and chronic effects. The main findings on DAG suggest that exercise did not change the total amount of DAG but induced the increase of DAG sn-2,3 and sn-1,3 (only DAG sn-1,2 affects insulin sensitivity) and may contribute to the complexity of the "athlete paradox" (Amati et al., 2011; Bergman et al., 2018). Acutely, after exercise, ceramide levels show an increase that rapidly returns to the baseline values or even lower after a single exercise bout (Bergman et al., 2016). LCA-CoA and acylcarnitine also did not change or slightly increase their levels after an exercise session (Thyfault et al., 2010), thus, there is no certainty to claim that exercise positively impacts lipid intermediates.

Inflammation

Behind the pathophysiology of T2DM is a significant contribution of low-grade inflammatory state due to apoptotic events on adipocytes (Petersen & Shulman, 2018). In those cases, exercise can have a strong anti-inflammatory effect (Pedersen, 2017). Muscle, apart from contractile function, works as an endocrine organ because it can secrete myokines with anti-inflammatory effects (Pedersen & Febbraio, 2008). Acutely, exercise promotes IL-6 secretion via muscle contraction, which is strongly linked to duration, intensity, and type (positive correlation with muscle mass recruitment) (Pedersen, 2017). Alongside the anti-inflammatory effects of IL-6, its release is also responsible for promoting downstream that leads to an increase in IL-1 receptor antagonist and IL-10 production (Magalhães et al., 2022). These anti-inflammatory myokines inhibit pro-inflammatory cytokines such as IL-1β and TNF-α (Pedersen, 2017). With regular exercise, IL-6 concentrations decrease due to training adaptations (Pedersen, 2017). However, the role of exercise as an anti-inflammatory agent does not end in IL-6 action; exercise also negatively impacts the infiltration of Monocyte chemoattractant protein-1 due to the reduction of visceral adipose tissue and consequently reduces the concentration of IL-1β and TNF-α (Magalhães et al., 2022; Trøseid et al., 2004). Besides the Monocyte chemoattractant protein-1 negative impact by IL-6 action, exercise also reduces the expression of receptors present on the monocyte membrane, affecting the inflammatory downstream that ends in cytokine production (Stewart et al., 2005). Some other myokines, such as brain-derived neurotrophic factor and IL-15, contribute to a reduction of white adipose tissue and an increase in fat and glucose oxidation, respectively (Magalhães et al., 2022; Pedersen & Febbraio, 2008).

Insulin Sensitivity

As explored before, the primary contributors to insulin resistance are lipotoxicity, inflammation, mitochondrial dysfunction, and hyperglycemia. These sources trigger complex molecular mechanisms, such as changes in the serine/threonine phosphorylation ratio of insulin receptor substrates, which disrupt the tyrosine phosphorylation required for effective insulin signaling(Boucher et al., 2014). The resultant metabolic dysfunction is evident in key insulin-responsive tissues, including adipose tissue, skeletal muscle, and the liver, where glucose uptake, utilization, and storage are impaired. Additionally, the stress induced by these factors extends to pancreatic β-cells, leading to their dysfunction and eventual loss, further exacerbating hyperglycemia and driving the progression of T2DM (Kasuga, 2006).

As explored in the previous chapters, exercise can be a key part of the treatment or a strategic tool to improve these metabolic processes. Resuming exercise enhances glucose delivery by its capacity to increase blood flow and promote improvements in artery structure, facilitating glucose delivery to the cells (Sylow et al., 2017). Exercise enhances glucose transport through the insulin-independent activation of GLUT-4 translocation via AMPK (Sylow et al., 2017). It also supports glucose metabolism by indirectly activating hexokinase 2 and promoting mitochondrial biogenesis through proliferator-activated receptor-coactivator 1α activation, thereby improving glucose regulation and metabolic flexibility (Sylow et al., 2017). Beyond that, exercise has the potential to decrease visceral adipose tissue and consequently decrease the inflammatory state by reducing pro-inflammatory cytokines (such as IL-1β, TNF-α) and increasing anti-inflammatory myokines (IL-6, IL-1 receptor antagonist, IL-10, IL-15, BDNF)(Magalhães et al., 2022). In turn, this will reduce the FFA that is released into the bloodstream due to improved visceral adipose tissue insulin sensitivity (Magalhães et al., 2022). All those processes result in less FFA, along with a reduction in glycemia, so less effort by the pancreatic β-cells to ensure the insulin needs, contributing to its health and normal function (Magalhães et al., 2022).

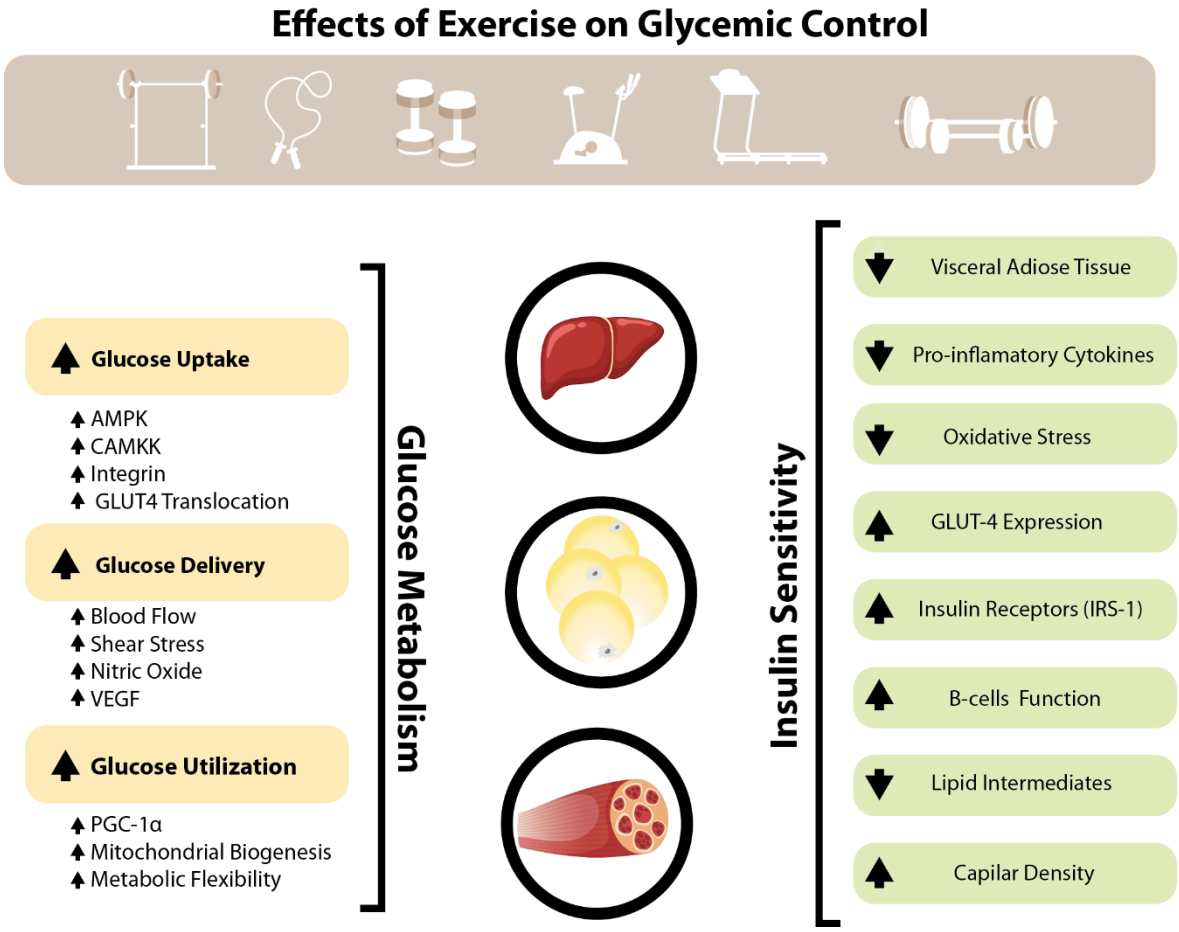

Figure 1. Effects of Exercise on Glycemic Control

1.3.3. Exercise, circadian rhythm and glycemic control in T2D

People with T2DM have also been associated with circadian clocks misaligned (Gabriel & Zierath, 2019), leading to increased endogenous glucose production during the night (dawn phenomenon) and contributing to a decreased morning glucose tolerance (Monnier et al., 2013). Although medication has developed over the past years, it cannot completely mitigate the dawn phenomenon, and exercise is an alternative strategy to minimize morning glucose levels (Peng et al., 2022). However, some people tend to respond differently to the same exercise dose, and we call this phenomenon “interindividual variability” (Ross et al., 2019). Therefore, for the same exercise program, it is possible to have a group of people who improve their cardiometabolic health and another group who are low-responders to exercise (Ross et al., 2019). The same occurs with people with T2DM; the objective is to enhance glucose metabolism, and some people tend to improve their glycemia, resulting in lower HbA1c, while others do not (Solomon, 2018). Different sources of inter-individual variability, such as genetics and epigenetics, cannot be controlled (Solomon, 2018). In fact, studies published by our group and others have found that exercise interventions at the

mean level can be considered ineffective in glycemic control (Liu et al., 2019; Magalhães et al., 2019). Those results demonstrate that interindividual variability is a real issue when prescribing exercise to people with T2DM; however, some of these factors can be controlled, like exercise type, intensity, volume, anti-hyperglycemic drugs and, more recently, exercise timing (morning, afternoon, evening), or exercise-meal timing (before/after meals) (Solomon, 2018). When it comes to exercise timing, recent intervention investigations support the fact that afternoon exercise can be more effective than morning exercise on glycemic control in people with T2DM and impaired fasting glucose (Kanaley et al., 2023; Menek & Budak, 2022; Savikj et al., 2019; Savikj et al., 2022). In 2019, eleven men with T2DM, nine taking metformin and 2 taking dietary treatment, were submitted to two weeks (three sessions per week) of morning or afternoon HIIT separated by two weeks of washout periods followed by two weeks of detraining period (Savikj et al., 2019). The investigators concluded that afternoon HIIT proved to be more effective than morning HIIT in enhancing blood glucose control in men with type 2 diabetes and, interestingly, morning HIIT had an immediate adverse effect, leading to an increase in blood glucose levels (Savikj et al., 2019). More recently, Kanaley and co-workers, in 2023, conducted a study to evaluate the postprandial glucose fluctuations during the overnight period after a day of standardized meals in individuals with obesity and those with both obesity and impaired fasting glucose, taking at least one glucose-lowering medication (Kanaley et al., 2023). They concluded that afternoon exercise decreased glucose levels in people with obesity and impaired fasting glucose during the night (Kanaley et al., 2023). However, Teo and co-workers conducted a 12-week combined training RCT to understand whether morning vs. afternoon exercise could be more effective on glycemic control(Teo et al., 2020). The study included 40 sedentary overweight adults, 20 with T2DM and 20 without (Teo et al., 2020). The results suggested that the exercise intervention improved glycemic control in people with T2DM, but they did not observe any differences between morning and afternoon exercise (Teo et al., 2020). Additionally, some observational (Hetherington-Rauth et al., 2022) and retrospective (Mancilla et al., 2021)studies have reported the same response trend to afternoon exercise on glycemic control.

2. Thesis purpose

Based on the available evidence, exercise may significantly impact glycemia when performed away from the dawn phenomenon (when insulin sensitivity is decreased). However, the studies reviewed have primarily focused on comparing morning versus afternoon exercise (Kanaley et al., 2023; Savikj et al., 2019; Teo et al., 2020) while using a

crossover design or an RCT approach. Additionally, most studies were conducted with male participants, limiting the generalization of the findings to the wide range of type 2 diabetes population. Furthermore, the evidence remains fragmented, as it fails to integrate other important confounding factors, such as medication, sedentary behavior, recreational PA, and sleep.

This thesis aims to expand on the current body of research regarding exercise and type 2 diabetes mellitus (T2DM) by implementing a 2-week randomized crossover intervention, with exercise performed at three separate times of day (morning, afternoon, and evening). The primary goal is to explore how exercise timing affects the 22-hour total area under the curve (AUC_{total}) for glucose levels. Our initial hypothesis lies with afternoon exercise having superior effects on glycemic control compared to the remaining periods in people with T2DM.

Chapter 2-The EX-Timing Project: Evaluating morning, afternoon, and evening exercise on the circadian clock in individuals with type 2 diabetes —a randomized crossover study protocol

Introduction

The global prevalence of diabetes has risen significantly over recent decades, currently affecting approximately 537 million adults worldwide (Magliano et al., 2021). This trend is projected to continue, with prevalence estimates reaching 643 million by 2030 and 783 million by 2040 (Magliano et al., 2021). Consequently, type 2 diabetes mellitus (T2DM) has become a major public health challenge, imposing a substantial financial burden on healthcare systems, with current global expenditures surging to 966 billion dollars (Magliano et al., 2021). Exercise can mitigate some of the harmful effects related to T2DM by improving several risk factors and by improving overall glycemic control (American Diabetes Association, 2023; Magalhães et al., 2022; Pedersen, 2017). However, some individuals struggle to improve their glycemic control and other health-related outcomes after an exercise intervention (Ross et al., 2019). These low-responders can be due to various sources of inter-individual variability in glycemic response, such as genetic and epigenetic factors, exercise adherence, sedentary time, exercise type, intensity, volume, anti-hyperglycemic drugs, and, more recently, exercise timing (Solomon, 2018).

Out of the different sources of interindividual variability, exercise timing has been identified as a current hot topic for T2DM (Gabriel et al., 2021; Gabriel & Zierath, 2019; Mason et al., 2020). This question is of critical importance since glucose metabolism can be under circadian influence, and exercise is key to controlling the expression of genes that influence human biological clocks (Gabriel & Zierath, 2019; Mancilla et al., 2021). On this topic, most evidence on the impact of exercise timing on glycemic control derives from animal model studies. However, there are three experimental studies focused on understanding which period of the day exercise can be more effective in glycemic control for T2DM (Kanaley et al., 2023; Savikj et al., 2019; Teo et al., 2020). Two trials suggest that the afternoon can be the most effective period of the day to improve glycemic control (Kanaley et al., 2023; Savikj et al., 2019), whereas one found no results on the effect of time of the day (Teo et al., 2020). Moreover, none of these previous investigations accounted for possible confounding factors such as sedentary time, recreational PA, or sleep duration. To overcome the shortcomings in the literature, this investigation aimed to understand whether exercise performed at different times of the day is more effective in glycemic control in people with T2DM.

Methodology

Recruitment process

This study represents a preliminary data analysis collected from an ongoing clinical trial (EX-TIMING), registered in the Clinical Trials (ClinicalTrials.gov Identifier: NCT06136013). More detailed information of the protocol can be found in the original paper (Magalhães et al., 2024). The study has been approved by the ethics committee of the Associação Protectora dos Diabéticos de Portugal (APDP), with the approval number 014/2023, and it will be conducted in accordance with the Declaration of Helsinki for Human Studies. Recruitment was performed through the APDP clinic and other sources, including media advertisements, referral networks, brochures, posters, and nearby health centers. An initial telephone screening was conducted to assess eligibility. Those who met the criteria were invited to an orientation session where detailed information about the study was provided. Written informed consent was obtained from all participants, and they were told that their participation was entirely voluntary and that they could withdraw at any time.

The inclusion criteria for this study comprised: 1) individuals with a prior diagnosis of T2DM based on the American Diabetes Association criteria; 2) currently on metformin, SGLT2, and GLP-1 medication; 3) aged between 55 and 75 years; 4) able to engage in exercise sessions. Participants were excluded if they were taking any other antihyperglycemic medications, had microvascular or macrovascular complications from T2DM, or could not provide informed consent. No direct or indirect incentives were offered to the participants.

Sample power calculations were performed to detect a predicted mean difference in 24-h AUC CGM-based glucose of 1.0 mmol/L with a within-patient SD of 0.85 mmol/L considering a type I error of 5%, a power of 80%, and a 30% dropout rate (Little et al., 2011). The sample calculations yielded a sample size of 17 individuals with T2DM, since this is a preliminary study, we were only able to include 9 participants. The selection and recruitment process are shown on the flow chart (Figure 2).

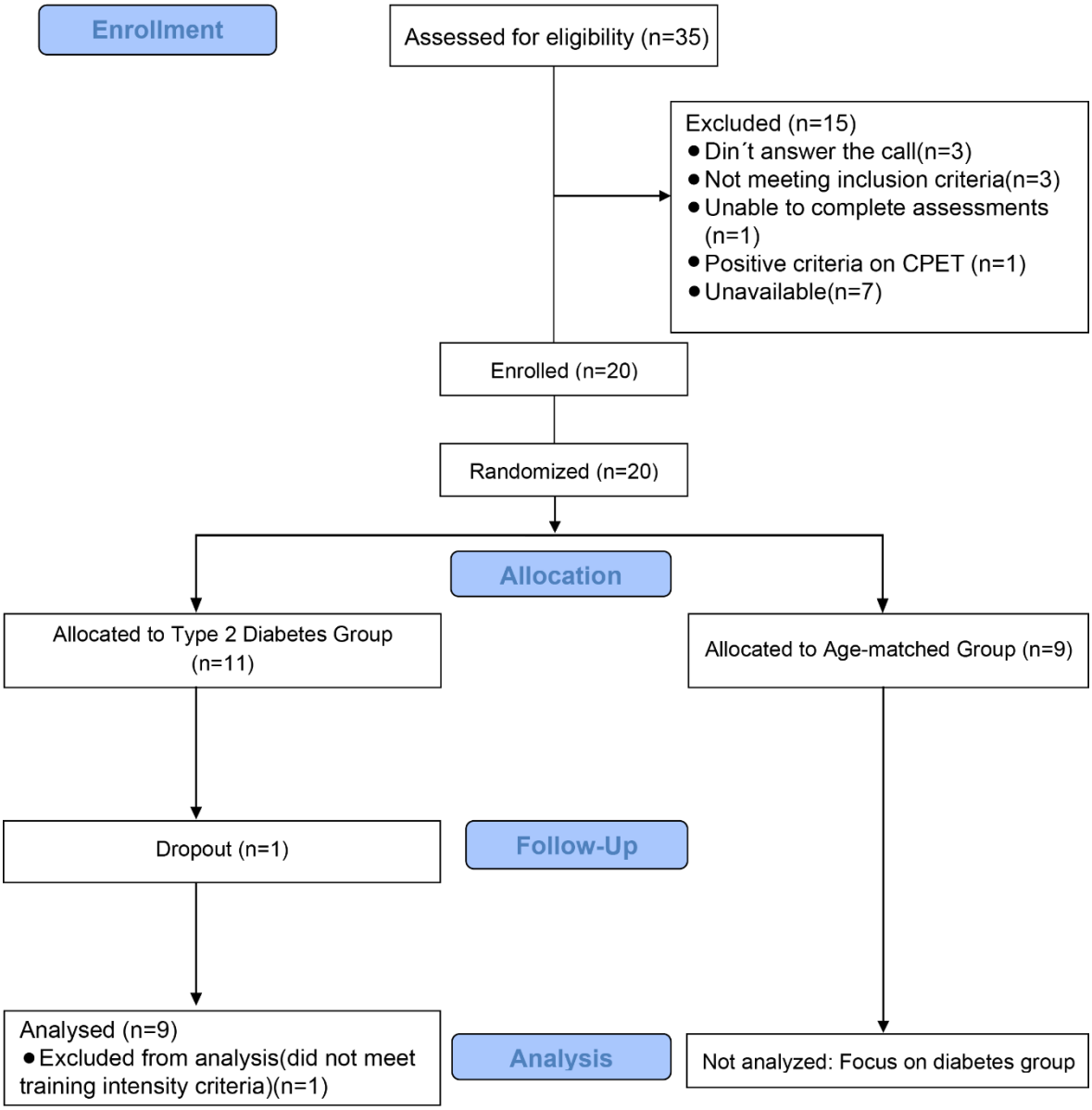

Figure 2. Study Flow Chart

Study design

All the participants will undergo an exercise intervention with three distinct training periods (i.e., morning, afternoon, and evening) while using a crossover design (FIGURE 3.). Our crossover design enables each participant to act as their own control, accounting for biological variability within individuals and their unique chronotypes, such as sleep and wake cycles. The exercise intervention was designed to span three months for each participant, with each training block (i.e., morning, afternoon, and evening) spanning 2 weeks with 6 supervised exercise sessions. After each training block, participants

underwent a 2-week washout period during which they were instructed to refrain from structured exercise training. During this washout phase, participants were asked to use their smartphone step counter to monitor their physical activity levels. The target daily step range was established based on the activity levels recorded through accelerometry before the intervention, ensuring that compensatory physical activity during the washout period was minimized. During the washout period (between blocks), participants continued using CGM and accelerometer for the entire 14-day period. All assessment protocols will be detailed in the following sections.

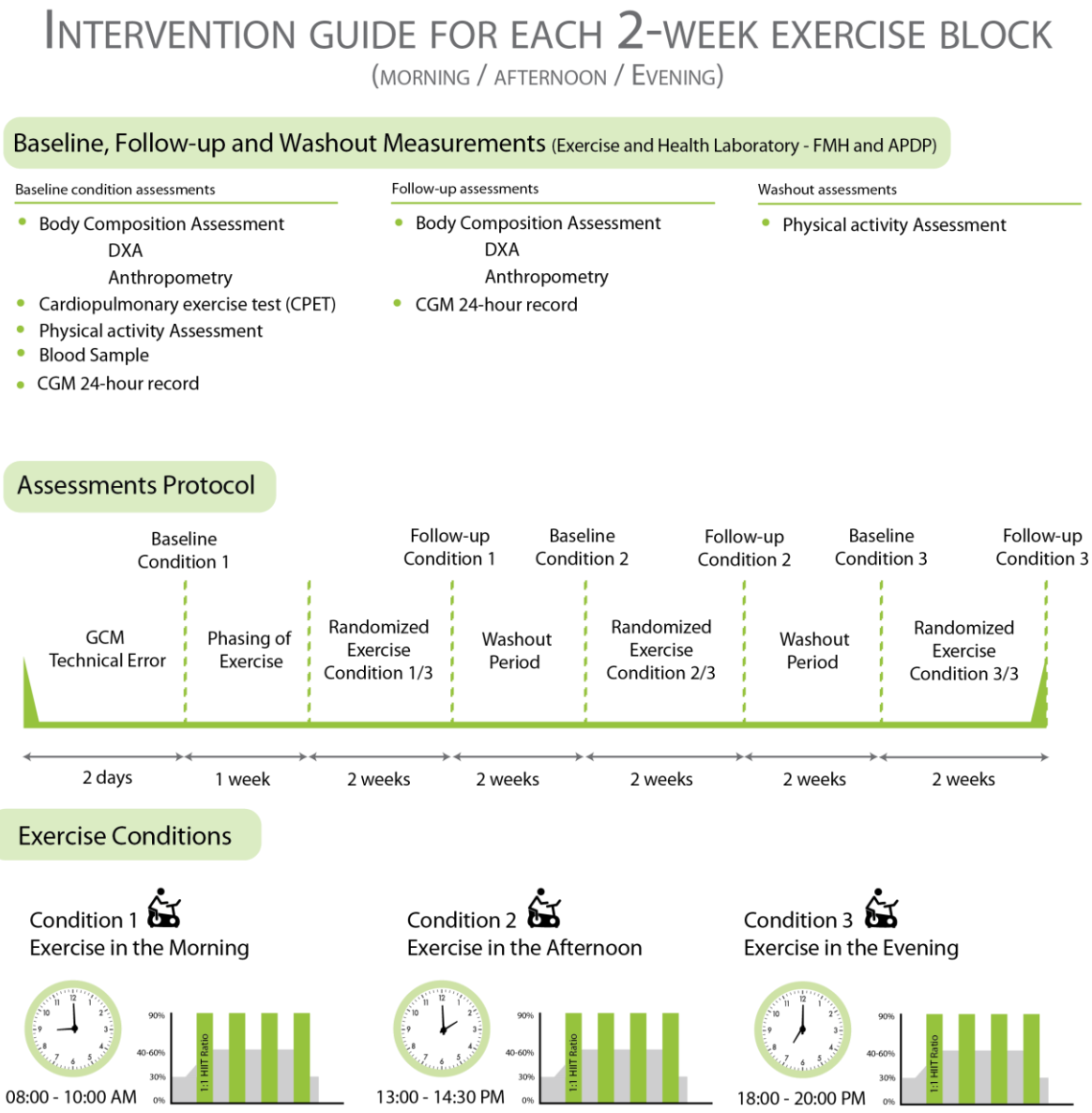

Figure 3. Study Design

Exercise intervention and phasing

Recognizing that many participants might not have experience with regular exercise, particularly at the high-intensity levels required for High-Interval Training (HIIT), we implemented a gradual familiarization phase during the first week before commencing the intervention. During Phase 1 (1st session), participants engaged in continuous moderate-intensity exercise, targeting 40–60% of their peak power output, as determined by cardiopulmonary exercise testing. In Phase 2 (2nd session), we introduced HIIT progressively, beginning with 2-minute intervals at 70% of their peak power output, followed by 1 minute at 40–60%. Finally, in Phase 3 (3rd session), the intensity increased to 80% of their peak power output, with subsequent recovery periods of 1 minute at 40–60%.

Following this preparatory week, participants commenced their first training block (scheduled for morning, afternoon, or evening) under the full HIIT protocol. This consisted of 1 minute of exercise at 90% of their peak power output, followed by 1 minute of rest at 40–60%, performed on a cycle ergometer. Each HIIT session was closely monitored using heart rate sensors (Polar H-10, USA) to ensure adherence to the prescribed intensity and to measure energy expenditure throughout the exercise.

The exercise prescription was tailored to each participant's body weight and adhered to physical activity guidelines to achieve a weekly energy expenditure target of 10 kcal/kg. The duration of each exercise session was adjusted based on individual weight and VO2peak and updated at the start of each intervention block.

Baseline, follow-up, and washout measurements

In this project, participants were evaluated six times: three at the beginning and three at the end of each training block. At the start of each block, assessments included the glycemic response to a standardized meal using continuous glucose monitoring (CGM), body composition through dual-energy X-ray absorptiometry (DXA), anthropometric measurements, cardiorespiratory fitness through a cardiorespiratory test, physical activity and sleep via accelerometry, fasting glucose through blood analyses, and HbA1c. All evaluations were repeated at the end of each block except for the cardiorespiratory test. The standardized meal test at the end of each training block was conducted on the same day as the last training session.

Body Composition

Participants were weighed to the nearest 0.01 kg, wearing minimal clothing and without shoes. Height was measured to the nearest 0.1 cm using a digital scale equipped with an

integrated stadiometer (Seca, Hamburg, Germany). Body mass index (BMI) was calculated by dividing weight (kg) by the square of height (m). Total and regional fat mass, lean body mass, bone mineral content, and bone mineral density were estimated using dual-energy X-ray absorptiometry (DXA) (Hologic Explorer-W, fan-beam densitometer, QDR for Windows software version 12.4, Waltham, USA). A whole-body scan was performed, measuring the attenuation of X-rays pulsed between 70 and 140 kV, synchronized with the line frequency, for each scanned image pixel. Abdominal and gynoid fat mass were assessed through partial analyses of the DXA scan, focusing on regions of interest predefined in the DXA settings. Following the manufacturer's protocol, a phantom containing six acrylic and aluminum fields of varying thicknesses and known absorptive properties were scanned with each participant as an external standard for tissue component analysis. A single laboratory technician was responsible for positioning participants, performing scans, and conducting analyses in accordance with the operator's manual and the standard analysis protocol.

Blood Samples

Fasting blood samples were collected at baseline and after each 2-week exercise intervention block. The samples were drawn in a seated position from the antecubital vein, following an overnight fast, into dry tubes and tubes containing ethylenediamine-tetraacetic acid (EDTA), an anticoagulant. Biological samples were centrifuged at 500 g at 4°C for 15 minutes, and the resulting plasma samples were stored at -80°C for later analysis. The blood samples were transported on ice to the APDP laboratory for glucose and insulin analysis. Each sample was utilized once for analysis and disposed of according to APDP clinical protocols. Serum glucose was measured using a colorimetric enzymatic assay with an automated analyzer (Olympus AU640, Beckman Coulter).

Cardiorespiratory fitness

Cardiopulmonary exercise testing (CPET) was used to evaluate maximal aerobic capacity and peak power output (PPO) and to screen for underlying cardiovascular conditions. To determine these parameters, participants underwent an individualized ramp incremental protocol to exhaustion on a cycle ergometer (Monark 839E, Kroons Vag, Sweden). The protocol began with an initial workload of 20 Watts/min, with subsequent increments of 5–20 Watts/min, adjusted based on individual cardiopulmonary responses during the first minute. Participants maintained a steady pedaling frequency of 60 revolutions per minute throughout the test. Under the supervision of a cardiologist, a 12-lead electrocardiogram

PC-based acquisition module continuously monitored participants while Omnia software recorded heart rate and additional parameters (Cosmed, Rome, Italy).

During the protocol, exhaled and inhaled gases were measured breath-by-breath using a gas analyzer (Quark RMR w/CPET, version 9.1, Cosmed, Rome, Italy). The highest 20-second value for oxygen uptake (VO_{2peak}) within the final minute of the exercise was recorded as the peak oxygen consumption (ml/kg/min), provided the participants reached volitional fatigue and met at least one of the following criteria: (1) a respiratory exchange ratio of 1.1 or greater, (2) attainment of the predicted maximum heart rate, or (3) no further increase in oxygen uptake despite an increase in workload. The PPO was identified as the workload reached during the final ramp stage completed by the participant. After completing the test, participants remained seated during the recovery phase. Blood pressure measurements were taken at one- and three post-exercise to monitor recovery dynamics.

Physical Activity, Sedentary behavior and sleep

Daily physical activity and sleep were assessed using ActiGraph GT9X accelerometers (ActiGraph, Pensacola, FL). Participants were instructed to maintain their usual physical activity patterns throughout the intervention period and to wear the accelerometer on their non-dominant wrist for the entire study duration. Device initialization and data retrieval were performed using Actilife® software (v.6.9.1; Fort Walton Beach, FL). Data processing was performed using the open-source R package GGIR (version 2.6), with non-dominant wrist cut-points for adults (light: 44.8 µg; moderate: 100.6 µg; vigorous: 428.8 µg) (Hildebrand et al., 2014). The accelerometer data yielded information on physical activity volume and intensity, including total minutes spent in light, moderate, and vigorous activity, total counts, and per minute. Additionally, it captured the time spent on sedentary behaviors and sleep.

Continuous glucose monitoring

The CGM sensor was placed subcutaneously on the back of the arm before participants consumed standardized pre-prepared meals designed to meet the recommended dietary guidelines for each meal type (breakfast, lunch, snack, or dinner). The reference values for calculating daily meals were 20% protein (Skurk et al., 2024), 55% carbohydrates (CHO), and 25% lipids (EFSA, 2017). The monitor CGM recorded interstitial glucose levels every 15 minutes and remained in place for 24 hours. During this period, all meals were standardized and provided to the participants. Additionally, the timing of all medications taken by participants was recorded. The CGM data were analyzed with MATLAB, using the trapezoidal method from a baseline concentration of zero to extract the AUC_{total} of the 22 hours of each day of standardized meals, starting right after the participant awakes. The

sleep time was defined by the report participants filled in during the first seven days and the accelerometry analysis. Mean glucose and time above target were also extracted from the analysis. Time above target was defined by the percentage of time above 180mg/dl.

Statistical Analysis

Descriptive analysis was conducted for the entire study sample, including mean ± standard deviation, as well as minimum and maximum values. Normal mixed models with random intercepts were applied to analyze changes in the following outcomes: AUC_{total}, mean glucose, weight and whole-body total fat. The models incorporated fixed effects for time and condition (three levels: morning, afternoon, and evening) and included the unique patient identifier as a random effect for all participants. Pairwise comparisons were also conducted to verify within-group changes. To analyze accelerometry data (sleep, sedentary behavior, light intensity physical activity, and moderate-vigorous physical activity) and cardiorespiratory fitness (VO_{2peak}), normality tests, including the Shapiro-Wilk and Kolmogorov-Smirnov tests, were performed to assess the distribution of the data. For data that followed a normal distribution, parametric tests (one-way ANOVA) were performed. For data that did not follow a normal distribution, non-parametric tests (Kruskal-Wallis) were used. Bonferroni post hoc testing was performed for multiple comparisons. These analyses were conducted with two software programs: STATA13 and IBM SPSS Statistics, version 30.0 (SPSS Inc., Chicago, Illinois).

Results

The baseline characteristics of the study population are summarized in Table 1. The cohort included 9 participants, predominantly male (7 males and 2 females), with a mean age of 65.8 ± 4.6 years. The mean duration since diabetes diagnosis was 9.3 ± 6.7 years. The mean body mass index (BMI) was 30.8 ± 3.4 kg/m², with 55.6% of the sample classified as overweight, 33.3% as obesity class I, and 11.1% as obesity class II. The mean value for glycated hemoglobin (HbA1c) was 6.5 ± 0.4%, with all participants having their HbA1c below the 7% threshold.

Table 1. Baseline characteristics of the participants

	Mean ± SD	Minimum	Maximum
Age, years	65.8±4.6	60	72
Males/Females, n	7/2		
Years since the diabetes diagnose	9.3±6.7	1	20
BMI, Kg/m^2	30.8±3.4	25.4	38
Body Composition			
WB FM, kg	30.7 ± 7.7	22.1	48.1
WB FM, %	37.6 ± 7.7	30.1	55.5
WB FMI, kg/m^2	11.5±3.7	7.7	20.6
WB LST, kg	50.6±7.3	38.6	60
WB LSTI, kg/m^2	18.6±1.7	16.5	21.3
Waist perimeter, cm	106.2±5.3	97.8	114.6
Cardiorespiratory Fitness			
VO_{2peak}, ml/kg/min	20.5±4.6	12.3	27.8
Glycemic control			
AUC_{total}	128.9±20.5	99.7	174.3
Mean Glucose, mg/dl	140.2±23.1	106.7	190
Time Above Target, %	15.3±11.4	2.5	39.1
Fasting Glycemia, mg/dl	111.8±19.4	82.5	133.8
HbA1c, %	6.5±0.4	5.71	6.91
Physical Activity and Sleep			
Sleep, min	400.8±46.3	331.7	479.4
SB, min	812.7±104	606.9	931.1
LIPA, min	133.2±49.6	77.1	231.9
MVPA, min	53.4±30.1	17	112.2
Medication for hypertension, %*	77.8		
IECA, %	42.9		
Beta-blockers, %	0		
Calcium Blockers, %	0		
Diuretics, %	42.9		
ARA, %	57.1		
Medication for Diabetes, %*	100		
Biguanides, %	77.8		
SGLT2, %	44.4		
GLP-1, %	22.2		

Abbreviations: WB, whole body; FM, fat mass; FMI, fat mass index; LST, lean soft tissue; LSTI, lean soft tissue index; VO_{2peak}, Peak Oxygen consumption; AUC_{total}, total area under the curve; HbA1c, glycated hemoglobin; SB, sedentary behavior; LIPA, light intensity physical activity; MVPA, moderate-vigorous physical activity; IECA, Inhibitors of the Enzyme Converting Angiotensin; ARA, Angiotensin II Receptor Antagonists.

*Percentage of those taking each type of medication; each participant can take more than one drug from each type.

Table 2 presents the data of the changes that occur between baseline and two weeks of intervention for each group, as well as the within and between-group changes. After adjusting for sex, age, and treatment order, there were no significant between-group changes in CGM for any of the training blocks AUC_{total} (MP vs. AP: β=-2.0; -18.8, 14.7); (MP vs. EP: β=0.04; -16.1,16.2); (EP vs. AP: β=-2.06; -18.6, 14.5) and mean glucose (MP vs. AP: β=-3.6; -21.2, 13.9); (MP vs. EP: β=-3.5; -20.4, 13.5); (EP vs. AP: β=-0.2; -17.5, 17.2). A significant time effect ($p<0.05$) was observed for within-group changes in AUC_{total} across all training periods. Pairwise comparisons for AUC_{total} revealed significant differences between the beginning and the end of each training period: -12.39±5,95 for the MP, -14.41±6,21 for the AP, and -12.35±5,73 for the EP. However, for the mean glucose levels, we only observed a within-group change for the AP (-13.04±6,49) and EP (-12.86±6,00) periods ($p<0.05$). Figure 4 represents individuals' AUC difference, and individuals' mean glucose difference between the baseline and the end of each training block with a significant time effect ($p<0.05$) for each training period, except for mean glucose in MP. There were no within and between-group changes in the body composition measures for the weight. A significant time effect ($p<0.05$) was observed for between-group changes in whole-body fat mass comparing MP with AP: β=-2.2; p=0.039. After running pairwise comparisons, there were no significant within-group changes, except for AP -1.8±0.74.

Table 3 presents the data from accelerometry and cardiorespiratory fitness across baseline and the two washout periods. For the different behaviors assessed throughout the intervention, we observed no significant differences across the time points for sleep ($p = 0.59$), sedentary behavior ($p = 0.97$), LIPA ($p = 0.84$), and MVPA ($p = 0.67$). Lastly, VO_2peak did not exhibit significant variation over time ($p = 0.97$).

Table 2. Glycemic control and body composition management at baseline and following 2 weeks for each intervention group: within and between group changes

	MP		AP		EP		MP vs AP	MP vs EP	EP vs AP
	BL	2 Weeks	BL	2 Weeks	BL	2 Weeks	β (95% CI)	β (95% CI)	β (95% CI)
AUC_{total}	135.6±22.2	125.7±12.4¶	129.1±20.6	117.1±27.4¶	126.2±20.3	113.9±27.9¶	-2.0 (-18.8; 14.7)	0.04 (-16.1; 16.2)	-2.1 (-18.6; 14.5)
Mean Glucose, mg/dl	146.3±23.1	138.7±13.9	140.3±22.9	128.7±28.9¶	140.2±22.0	127.3±27.0¶	-3.6 (-21.2; 13.9)	-3.5 (-20.4; 13.5)	-0.2 (-17.5; 17.2)
Weight, Kg	83.1±8.7	82.6±8.3	83.1±8.7	82.7±8.8	82.5±8.4	82.3±8.2	0.1 (-0.8; 1.0)	0.4 (-0.5; 1.3)	-0.3 (-1.2; 0.6)
WB TOT FAT; %	36.0±8.2	36.4±8.0	37.8±6.9	36.0±8.2¶	37.0±8.6	36.8±9.1	-2.2 (-4.2; -0.1) *	-0.6 (-2.6; 1.5)	-1.6 (-3.6; 0.5)

Abbreviations: MP, morning period; AP, afternoon period; EP, evening period; BL, baseline; AUC_{total}, total area under the curve; WB TOT FAT, whole body total fat.

¶ Within-group changes significant at $p < 0.05$

*Between-group changes significant at $p < 0.05$

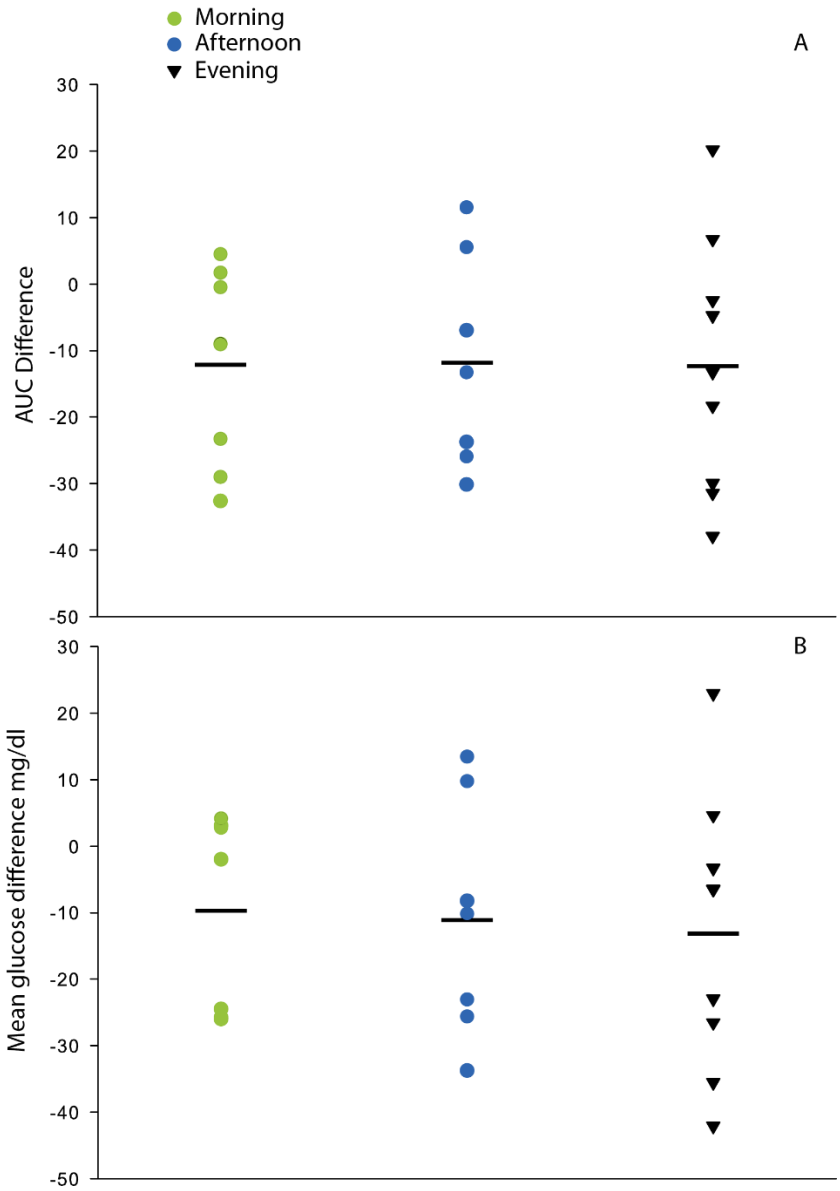

Figure 4. (A) CGM-based individuals' AUC and (B) mean glucose differences. Green dots represent each participant difference from baseline to the end of block for AUC_{total} (A) and mean glucose (B) in MP; Blue dots represents each participants difference from baseline to the end of block for AUC_{total} (A) and mean glucose (B) in AP; Black triangles represent each participants difference from baseline to the end of block for AUC_{total} (A) and mean glucose (B) in EP; Black horizontal lines represent the mean value of the difference. No significant ($p<0.05$) between-group changes occurred, neither on AUC_{total} nor Mean glucose levels. Within group changes occurred in the three groups for AUC_{total} ($p<0.05$), for the mean glucose, only MP did not show significant within-group changes.

Table 3. Physical activity, sedentary behavior, sleep and cardiorespiratory fitness at baseline, first washout and second washout: between-group changes

	BL	WO1	WO2	p-value
Sleep, min	400.8 ± 46.3	392.0 ± 71.2	419.1 ± 49.6	0.59
SB, min	812.7 ± 104	816.8 ± 97.9	805.5 ± 79.6	0.97
LIPA, min	133.2 ± 49.6	129.7 ± 45.6	120.4 ± 46.5	0.84
MVPA, min	53.4 ± 30.1	54.4 ± 30.9	42.8 ± 29.5	0.67
VO_{2peak}, ml/Kg/min	20.5 ± 4.6	21.0 ± 5.7	20.4 ± 5.7	0.97

Abbreviations: BL, baseline; WO1, first washout; WO2, second washout; SB, sedentary behavior; LIPA, light intensity physical activity; MVPA, moderate-vigorous physical activity; VO_{2peak}, peak oxygen consumption.

Discussion

This randomized crossover trial aimed to determine whether exercise is more effective for glycemic control in the morning, afternoon, or evening period. Our preliminary results suggest that there were no significant differences in AUC_{total} or mean glucose levels between exercises performed in either of the training blocks. Moreover, we observed a time effect for all the training blocks, suggesting that the exercise dose may be the main driver in glycemic control.

Exercise is a key component of T2DM treatment due to its ability to increase glucose delivery, transport, and metabolism in an insulin non-dependent pathway (Sylow et al., 2017). Consequently, exercise can improve insulin sensitivity due to increased capillarization, an increase in GLUT-4 translocation, improvements in mitochondrial function and lipid intermediaries, and increased hexokinase expression with an impact on glycogen storage (Sylow et al., 2017). However, not every person responds positively to exercise programs, and some tend to be “low-responders” (Solomon, 2018). This can be due to different sources of interindividual variability, such as exercise type, intensity, volume, meal timing, and medication (Solomon, 2018). However, more recently, questions about other sources of interindividual variability have been discussed, such as exercise timing and its impact on glycemic response. On this topic, we found no difference between performing exercise in the morning, afternoon, or evening on AUC_{total} and mean glucose levels. Still, the current body of literature suggests that afternoon exercise may be more advantageous for glycemic control in people with T2DM and those with impaired fasting glucose

(Hetherington-Rauth et al., 2022; Kanaley et al., 2023; Savikj et al., 2019). In one of these studies, Savikj and co-workers conducted an exercise protocol similar to ours, with the same exercise regimen (1:1 HIIT on a cycle-ergometer) and the same exercise intervention time frame (2 weeks of intervention). However, despite using the same clinical population (i.e., Individuals with T2DM), the authors focused only on men taking metformin as their solo T2DM medication (Savikj et al., 2019). Moreover, they only had two experimental blocks (morning and afternoon), whereas our protocol had three. All the aforementioned reasons could partly explain the different results between studies. In fact, our sample included both men and women on different T2DM medications, allowing for the results to be generalized to a wider audience of people with T2DM.

Another important topic lies with controlling other possible confounding variables during the intervention that could impact glycemic control. In our study, we controlled other sources of interindividual variability, such as diet, 24-hour movement variables, weight loss, and changes in cardiorespiratory fitness. For instance, at baseline and follow-up assessments, standardized meals were given for the entire day of each, ensuring that each participant had the same conditions in the three different periods. Beyond that, during the washout period, our participants were instructed to refrain from any structured physical activity and were instructed to use an accelerometer to record free-living physical activity, sedentary behavior, and sleep, all of which did not change during the study. Also, the cardiorespiratory fitness was maintained from baseline to the start of each exercise block, providing evidence that there was no carryover effect from possible gains in cardiorespiratory fitness to the next training block. All those interindividual sources of variability were not controlled in Savikj et al.´s study, raising the question of whether there are any changes in these confounding factors that could explain/bias their results. With similar results to the Savikj´s et al. study. Kanaley and colleagues conducted a crossover trial to understand the acute effects of exercise in the morning vs. afternoon on fasting glucose in people with obesity and impaired fasting glucose (Kanaley et al., 2023). They found that afternoon exercise decreased glycemia over the night, whereas no changes were observed in the glucose level at wake-up time (Kanaley et al., 2023). Our study focused only on the glucose AUC_{total} for a 22-hour period after wake-up time, and no sensitive analysis was performed during the night period. Moreover, Kanaley and colleagues' exercise protocol was performed on a treadmill while walking with people with obesity and impaired fasting glucose who are taking at least one anti-hyperglycemia drug. These differences in the type of clinical population and protocol may explain the difference between our results and theirs.

With a different study design, Teo and co-workers performed an RCT to determine if a 12b week multimodal exercise program during the morning or afternoon period was more effective in improving glycemic control (Teo et al., 2020). The findings from this investigation concluded that exercise elicits benefits in glycemic control without having any differences between morning and afternoon exercise periods (Teo et al., 2020). Despite having a different study design, these results are aligned with ours, which could provide some insights into the long-term effects of exercise timing on glycemic control. In fact, using an RCT design by Teo and co-workers was key in enabling a bigger time frame of intervention (12 weeks) while also allowing for the comparison of the intervention groups against controls.

When it comes to possible mechanisms of the impact of exercise timing on glycemic control, glucose metabolism seems to be under circadian influence, with insulin sensitivity presenting fluctuations throughout the 24-hour period (Mason et al., 2020). In general, healthy people tend to show an increased insulin sensitivity in the morning, compared to afternoon and evening (Mason et al., 2020). In contrast, people with T2DM have their insulin sensitivity compromised in the morning due to a misalignment of circadian rhythms and usually show a sudden morning glucose rise (dawn phenomenon) (Mason et al., 2020). The current evidence shows that exercise may be more effective when performed during the opposite period when the dawn phenomenon occurs (Hetherington-Rauth et al., 2022; Kanaley et al., 2023; Mancilla et al., 2021; Savikj et al., 2019). However, T2DM is a highly heterogeneous disease (Leslie et al., 2023), with individuals having distinct phenotypes based on different contributions from lifestyle risk factors and genetic predisposition. Thus, it is possible that those with an unfavorable body composition and non-alcoholic fatty liver derived from an unhealthier lifestyle could be more susceptible to exercise timing since this obese phenotype is one of the major causes of dawn phenomenon and hepatic insulin resistance. The inclusion of individuals with other differentiated T2DM phenotypes could represent a major source of interindividual variability that could partly explain the absence of significant results when comparing morning, afternoon, and evening. Future studies should consider the heterogeneity of T2DM and assess the implications of exercise timing on non-alcoholic fatty liver and hepatic insulin resistance.

On a different note, our results showed that there was a between-group effect for the whole-body total fat, favoring the afternoon training against the morning period. A recent review study has highlighted that morning exercise may be more effective in body composition

management when compared with other periods of the day (Blankenship et al., 2021). However, there are also other observational and retrospective investigations in people with T2DM, providing evidence that afternoon exercise is associated with improved body composition and fat oxidation (Hetherington-Rauth et al., 2022; Mancilla et al., 2021). But more importantly, our findings are still preliminary and warrant further corroboration from the remaining participants who have not yet finished the trial.

The strength of this study is its study design. Being a randomized crossover trial allows the same sample to perform exercise in three different periods of the day while discarding any biased configuring variables such as genetics and distinct chronotypes. Additionally, controlling all the confounding variables ensures that the changes in AUC_{total} and mean glucose were derived from the exercise intervention. Such an example lies with the 24-hour movement outcomes that were controlled throughout the study but also with the absence of weight loss throughout the intervention. Our limitations are mainly about the duration of the intervention; all the results are short-term, and long-term intervention in this field is still a gap in the literature. The last limitation is that each person can have their own chronotype and glucose excursion, which can be limited by using a specific time of the day to prescribe exercise. Thus, future studies should consider prescribing exercise based on the peak of glycemia, previously determined by CGM assessment.

Conclusion

The preliminary results from this study reveal that exercise effectively enhances glycemic control in people with T2DM without alterations in body composition, diet, free-living physical activity, and cardiorespiratory fitness. However, we did not observe significant changes in AUC_{total} and mean glucose between the three training periods (MP, AP, EP). These results shed some light on the exercise timing paradigm and may have practical implications for exercise physiologists and other healthcare professionals working with people with T2DM. Nevertheless, careful is advised since these results should be confirmed with the conclusion of the whole sample from the study.

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