International Doctoral Thesis / Tesis Doctoral Internacional

Diurnal variation of the effect of aerobic exercise on glucose regulation and energy metabolism in humans: role of sex

Variación diurna del efecto del ejercicio aeróbico sobre la regulación de la glucosa y el metabolismo energético en humanos: rol de sexo.

UNIVERSIDAD DE GRANADA

Programa de Doctorado en Biomedicina

Departamento de Fisiología

Facultad de Farmacia

Universidad de Granada

Directores de Tesis: Dr. D. Francisco Jesús Rodríguez Huertas y Dr. D. Francisco José Amaro Gahete

Raquel Sevilla Lorente

2024

Editor: Universidad de Granada. Tesis Doctorales
Autor: Raquel Sevilla Lorente
ISBN: 978-84-1195-543-0
URI: https://hdl.handle.net/10481/97399

Diurnal variation of the effect of aerobic exercise on glucose regulation and energy metabolism in humans: role of sex

Raquel Sevilla Lorente

2024

La doctoranda Dª. Raquel Sevilla Lorente ha realizado la presente Tesis Doctoral Internacional siendo beneficiaria de un contrato predoctoral del programa de Formación de Profesorado Universitario (referencia: FPU19/03745) del Ministerio de Universidades, por Resolución de 16 de octubre de 2019 de la Secretaría de Estado de Universidades, Investigación, Desarrollo e Innovación, en el Departamento de Fisiología de la Universidad de Granada.

Table of Contents

Research projects and funding

The present International Doctoral Thesis was performed under the framework of the DIVA STUDY (ClinicalTrials.gov ID: NCT05369715) and was funded by the following organizations:

- "Ayudas europeas a proyectos de investigación aplicada a la Actividad Física Beneficiosa para la Salud y la Medicina Deportiva" (EXP_77437).
- "Consejo Superior de Deportes, Plan de Recuperación, Transformación y Resiliencia", European Union, Next Generation EU.
- European Regional Development Funds (PID2021-126788OB-I00).
- Spanish Ministry of Science and Innovation (PID2022-141506OB-I00) and the European Regional Development Funds (ERDF).
- Spanish Ministry of Science, Innovation and Universities MCIN/AEI/10.13039/501100011033 (FJC2020-044453-I, RYC-2017-23013, PID2020-114054RA-I00 1001100482).
- Spanish Ministry of Universities (FPU 19/03745, FPU20/05530).
- University of Granada Plan Propio de Investigación-Excellence actions: Unit of Excellence on Exercise Nutrition and Health (UCEENS).

Declaration of Interest Statement

Authors from all the works presented in this International Doctoral Thesis declare that they have no conflict of interest associated with research, findings, or the publication of this work.

Data Availability

Any additional data that support the findings of this International Doctoral Thesis are available from R. Sevilla-Lorente upon reasonable request.

List of abbreviations

ACN - Acetonitrile

ADP - Adenosine Diphosphate

AMPK - AMP-activated protein kinase

ANOVA - Analysis of Variance

ARB - Angiotensin II Receptor Blocker

ARNTL - Aryl Hydrocarbon Receptor Nuclear Translocator-Like

ATP - Adenosine Triphosphate

BMAL1 - Brain and Muscle ARNT-Like 1

BMI - Body Mass Index

BNGE - Blue Native Gel Electrophoresis

CI - Confidence Interval

CIART - Circadian-Associated Repressor of Transcription

CK - Creatine Kinase

CNDP2 - Cytosolic Non-Specific Dipeptidase 2

CONSORT - Consolidated Standards of Reporting Trials

CREB/MAPK - Response element-binding protein / mitogen-activated protein kinase

CRP - C-Reactive Protein

CRY – Cryptochrome Circadian Regulator

CVD - Cardiovascular Disease

DBP - Diastolic Blood Pressure

dRM - Cohen's d for Repeated Measures

EDTA - Ethylenediaminetetraacetic Acid

EE - Energy Expenditure

ENMO - Euclidean Norm Minus One

FDR - False Discovery Rate

FCCP - Carbonyl Cyanide-p-Trifluoromethoxyphenylhydrazone

FGF21 - Fibroblast Growth Factor 21

GDF15 - Growth Differentiation Factor 15

GLUT4 - Glucose Transporter Type 4

GLP-1 - Glucagon-Like Peptide-1

HDL - High-Density Lipoprotein

HDL-C - High-Density Lipoprotein Cholesterol

HIIT - High-Intensity Interval Training

HLF - Hepatic Leukemia Factor

HÖME - Horne-Östberg Morningness-Eveningness

HOMA - Homeostatic Model Assessment

HRmax - Maximum Heart Rate

HRR - Heart Rate Reserve

IAM - Iodoacetamide

IBGI - Improved Basal Glucose/Insulin

IGF-1 - Insulin-Like Growth Factor 1

IL-6 - Interleukin 6

IPAQ - International Physical Activity Questionnaire

Lac-Phe - Lactate-Phenylalanine

LDH - Lactate Dehydrogenase

LDL - Low-Density Lipoprotein

MAPK - Mitogen-Activated Protein Kinase

MBP - Mean Blood Pressure

mRNA - Messenger RNA

N - Number of participants

NR1D1 - Nuclear Receptor Subfamily 1 Group D Member 1

NR1D2 - Nuclear Receptor Subfamily 1 Group D Member 2

OxPhos - Oxidative Phosphorylation

PER - Period Circadian Regulator

PPARα - Peroxisome Proliferator-Activated Receptor Alpha

PRISMA - Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PSM - Peptide-Spectrum Match

PYY - Peptide YY

Q-Q Plot - Quantile-Quantile Plot

Q1 - First Quartile

Q2 - Second Quartile

Q3 - Third Quartile

R - Correlation Coefficient

RER - Respiratory Exchange Ratio

RM - Repetition Maximum

RNA-Seq - Ribonucleic Acid Sequencing

Ror - Retinoic Acid-Related Orphan Receptor

ROS - Reactive Oxygen Species

RPE - Rating of Perceived Exertion

SBP - Systolic Blood Pressure

SC - Supercomplexes

SC/VDAC - Supercomplexes/Voltage-Dependent Anion Channel

SCN - Suprachiasmatic Nucleus

SD - Standard Deviation

SDS - Sodium Dodecyl Sulfate

T3 - Triiodothyronine

T4 - Thyroxine

TC - Total Cholesterol

TGL - Triacylglycerol Lipase

TEAB - Triethylammonium Bicarbonate

TG - Triglycerides

TSH - Thyrotropin

UHPLC-MS - Ultra High-Performance Liquid Chromatography-Mass Spectrometry

UI - International Unit

VAS - Visual Analogue Scale

VCO_2 - Volume of Carbon Dioxide Production

VO_2 - Volume of Oxygen Consumption

VO_2max - Maximum Volume of Oxygen Consumption

VO2 peak - Peak Oxygen Uptake

WASO - Wake After Sleep Onset

Abstract

Mammalian cells possess molecular clocks that control whole-body metabolism. These clocks are intricately related to cardiometabolic health, being its disruption linked to health disorders such as cardiovascular disease, type 2 diabetes, or obesity. Exercise, a well-known promoter of overall health, acts as a zeitgeber (or 'time-keeper') capable to reset molecular clocks, with potential variations based on sex.

This International Doctoral Thesis aimed to investigate the diurnal variation of exercise on glucose regulation and energy metabolism in men and women and, to elucidate the underlying molecular mechanisms within the skeletal muscle.

Overall, a single bout of exercise performed either in the morning or the evening does not influence the systemic effects on blood pressure, glucose regulation, energy metabolism and perceived appetite feelings (Chapter 3, 4, and 5). However, the molecular changes in the skeletal muscle after moderate-intensity aerobic exercise show an evident sexual dimorphism and time-of-day differences. In women evening exercise results a stronger stimulus than morning, with higher reliance on carbohydrates in the skeletal muscle, increased cytokine expression, and increased potential to suppress appetite in the short-term. Contrary, in the evening the molecular response in men promotes mitochondrial function while inhibiting carbohydrate utilization, although time-of-day variations are less evident than in women (Chapter 4 and 5).

Findings from this Thesis highlight the importance of considering sex-specific differences when designing exercise interventions. A single bout of moderate-intensity exercise was not enough to extrapolate the sex and time-of-day-dependent molecular changes to systemic effects. However, molecular changes may translate into observable systemic effects with higher exercise-intensity or chronic exercise interventions. Questions remain unanswered regarding the effect of other exercise protocols (e.g., higher intensity or resistance training), the long-term effect or the response on patients with cardiometabolic disorders.

This International Doctoral Thesis provides a detailed molecular framework helpful for future research on precise exercise prescriptions in clinical settings.

Resumen

Las células de los mamíferos poseen relojes moleculares que regulan el metabolismo a nivel de todo el organismo. Estos relojes están íntimamente relacionados con la salud cardiometabólica, estando su alteración vinculada a trastornos como las enfermedades cardiovasculares, diabetes tipo 2 u obesidad. El ejercicio, un bien conocido promotor de la salud en general, actúa como un zeitgeber (o 'marcador de tiempo') capaz de restablecer los relojes moleculares, con posibles variaciones dependientes del sexo.

El objetivo de esta Tesis Doctoral Internacional fue investigar la variación diurna del ejercicio en la regulación de la glucosa y el metabolismo energético en hombres y mujeres, y elucidar los mecanismos moleculares subyacentes en el músculo esquelético.

En general, una sola sesión de ejercicio realizada por la mañana o por la tarde no influye en la respuesta sistémica de la presión arterial, la regulación de la glucosa, el metabolismo energético y la percepción del apetito (Capítulo 3, 4 y 5). Sin embargo, las adaptaciones moleculares en el músculo esquelético después de una sesión de ejercicio aeróbico de intensidad moderada muestran un evidente dimorfismo sexual y diferencias según la hora del día. En las mujeres, el ejercicio vespertino resulta ser un estímulo más intenso que el matutino, con una mayor dependencia de los carbohidratos en el músculo esquelético, una mayor expresión de citocinas y un mayor potencial para suprimir el apetito a corto plazo. Por el contrario, en los hombres, la respuesta molecular vespertina promueve la función mitocondrial mientras inhibe la utilización de carbohidratos, aunque las variaciones entre la mañana y la tarde son menos evidentes que en las mujeres (Capítulo 4 y 5).

Los hallazgos de esta tesis resaltan la importancia de considerar las diferencias específicas de sexo al diseñar intervenciones de ejercicio. Una sola sesión de ejercicio de intensidad moderada no fue suficiente para reflejar los cambios moleculares dependientes del sexo y la hora del día en efectos sistémicos. Sin embargo, los cambios moleculares podrían traducirse en efectos sistémicos observables con una mayor intensidad de ejercicio o intervenciones de ejercicio crónico. Quedan preguntas sin responder sobre el efecto de otros protocolos de ejercicio (por ejemplo, mayor intensidad o entrenamiento de fuerza), el efecto a largo plazo o la respuesta en pacientes con trastornos cardiometabólicos.

Esta Tesis Doctoral Internacional proporciona un marco molecular detallado, útil para futuras investigaciones centradas en la prescripción precisa de ejercicio en entornos clínicos.

GENERAL INTRODUCTION

Chapter 1. Circadian physiology: a perspective from cardiometabolic parameters and exercise

1. Mammalian cells possess molecular clocks

In our natural environment, the rotation of Earth results in predictable changes in light and temperature. Accordingly, evolution has favored the development of circadian or biological clocks. Derived from the Latin *circa* (meaning 'about') and *dies* (meaning 'day'), these are endogenous cellular mechanisms which allow living systems to track time. Endogenous clocks provide a survival advantage by enabling organisms to anticipate daily environmental changes and align their behavior and physiology with the appropriate time-of-day[1].

In animals, circadian clocks can be considered as an integrated system, beginning with genes that ultimately lead to behavioral outputs. The circadian system regulates the daily sleep and wake cycle – which is perhaps the most obvious function –, but also additional and numerous physiological processes including body temperature, eating behavior, hormone secretion or glucose homeostasis, among others[2].

The circadian system is exquisitely accurate and the largest known regulatory network in normal physiology. Importantly, disruptions in circadian cycles can lead to notorious disorders across various physiological processes which finally increase the risk of cardiovascular disease, type 2 diabetes or obesity[3]. The prevailing characteristics of modern societies including exposure to artificial lights, shifted sleep patterns, inadequate eating windows or sedentarism, contribute to humans increasingly ignoring natural circadian cues[4]. Thus, to better understand the disease process and to identify new targets for treatments, it is imperative to precisely define the interaction between our circadian system, physiology, and lifestyle.

1.1. Cell-autonomous circadian clock

Even in the absence of light-dark environmental cues physiological circadian rhythmicity persists. Most circadian oscillations therefore are not responses to the light-dark cycle, instead, they are generated by an internal clock: the core clock[5].

In animals, the core clock is a cell-autonomous transcriptional-translational feedback loop present ubiquitously in almost every cell in the body[6]. Clock genes compose the loop, including *Clock*, *Bmal1*, *Period* (*Per1*, *Per2* and *Per3*), and *Cryptochrome* (*Cry1*, *Cry2*). *Clock* and *Bmal1* are the transcriptional activators that positively regulate the expression of *Per1, Per2, Per3, Cry1 and Cry2*. *Per* and *Cry* products accumulate and form a complex which

interacts with *Clock* and *Bmal1,* repressing their own transcription. Other genes play additional roles in the circadian loop, such as *Rev-erb* (-α and -β), and *Ror* (-α,- β,and -γ). *Rev-erb* and *Ror* influence the transcription of *Bmal1* (and *Clock* to a lesser extent) by, respectively, acting to repress and activate their expression (Figure 1). All of these transcriptional events generate in cells endogenous cycles of approximately 24 hours in mRNA and protein levels[2].

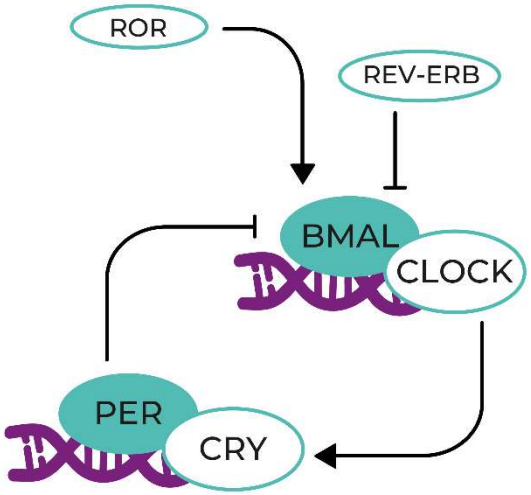


Figure 1. The core clock transcriptional-translational feedback loop (5). Clock and Bmal1 positively regulate the expression of Per1, Per2, Per3, Cry1 and Cry2. Per and Cry products form a complex which repress Clock and Bmal1. Rev-erb (-α and -β), and Ror (-α,- β,and -γ) influence the transcription of Bmal1 (and Clock to a lesser extent) by, respectively, repressing and activating their expression.

1.2. Tissue organization

Approximately 20.000 neurons composing the hypothalamic suprachiasmatic nucleus (SCN) play the central role for the generation of circadian rhythms[7]. Peripheral non-SCN cells also possess autonomous circadian oscillators, all of them also conserving the core clock machinery but significantly differing in the relative contribution of the individual clock components, the manner in which they reset, and the output pathways that are under their control. To ensure the effective functioning of circadian physiology, molecular central and peripheral clocks need to accurately track time and adapt to environmental cues. The SCN receives direct photic input from the retina and convey signals to light insensitive peripheral clocks through autonomic innervation, body temperature and endocrine signaling (e.g., glucocorticoids) (Figure 2)[2]. Under natural light-dark

conditions, bright light strongly inhibit melatonin production[8] and promotes corticosterone release in the adrenal gland[9]. Corticosteroids promote arousal, alertness, and catabolic metabolism is muscle and adipose tissue[3]. Sleep-arousal, thermoregulation, and hunger-satiety centers in the hypothalamus are intimately integrated with local SCN outputs[7]. Further, external cues known as zeitgebers ('time-keepers') also regulate tissue-specific clocks via local signals derived from cellular metabolism. Examples of zeitgebers are food, light, heat and exercise which are able to partially replace the time-communicating role of the SCN in liver, pancreas, or skeletal muscle among other tissues (Figure 2)[3].

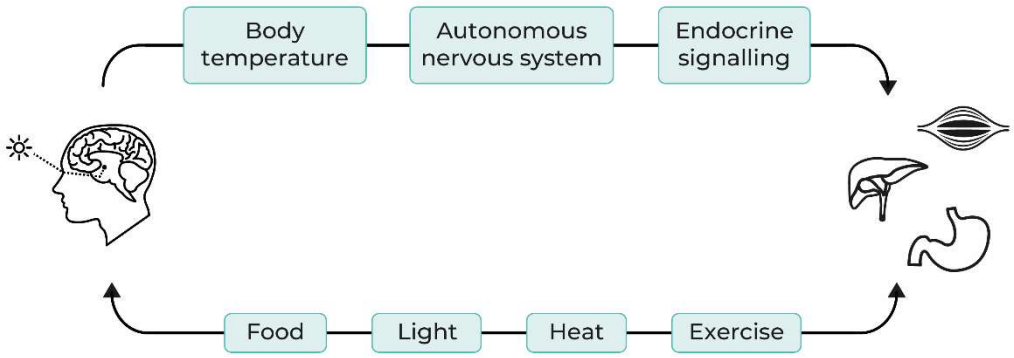


Figure 2. Central and peripheral clocks organization[2,3] The central clock in the SCN receives timing information through direct photic input from the retina and convey signals to light insensitive peripheral clocks (e.g., skeletal muscle, liver, or stomach) through body temperature autonomic innervation and endocrine signaling. Simultaneously, timing information from zeitgebers (e.g., food, light, heat, or exercise) are able to partially replace light cues.

In summary, the circadian machinery of each mammalian cell integrates the core clock loop, cellular metabolism, and extracellular systemic cues in order to ultimately regulate tissue function (Figure 3).

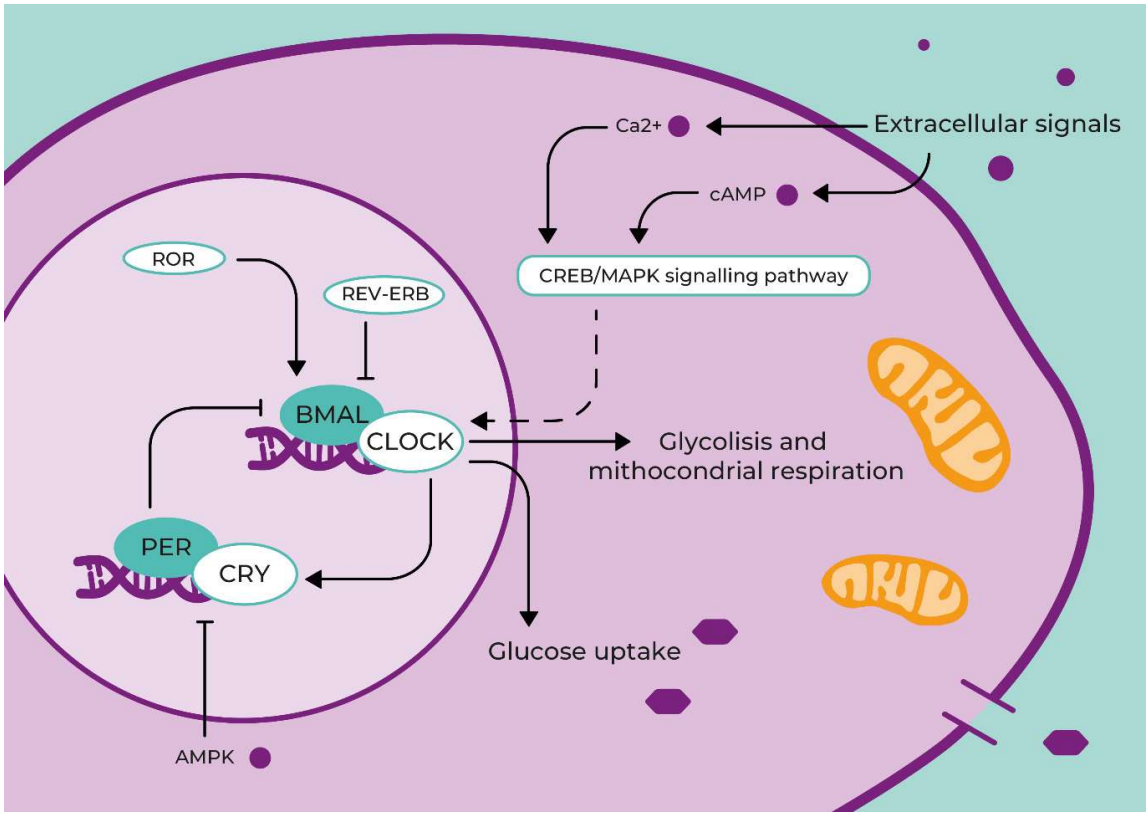


Figure 3. Integrated overview of the circadian machinery (skeletal muscle cell) [5,15]**.** The core clock machinery of every mammalian cell interacts with cellular metabolism and extracellular systemic cues to regulate tissue function. The skeletal muscle cell is here used as an example. CREB/MAPK signaling pathway (a key pathway for signal transduction in response to growth factors, hormones, and stress) has an implication in circadian periodicity. Dashed lines indicate implication but not necessarily direct activation of BMAL and CLOCK. AMPK (a key energy sensor in skeletal muscle) activity reduces the stability of CRY1 and PER2. BMAL1 promotes glucose uptake, glycolysis and mitochondrial respiration.

2. Circadian machinery orchestrates cardiometabolic health

While in humans its complexity is less understood compared to animal models, circadian rhythms have been clearly identified in multiple aspects of cardiometabolic health (Figure 4). Previous evidence has reported that blood pressure, heart rate, endothelial function, or thrombus formation are under circadian clock's influence in humans[10]. Beyond, the intimate and reciprocal interaction between circadian clocks and fundamental metabolic pathways highlights its pivotal role in health maintenance. Circadian rhythmicity is present in glucose and insulin dynamics, glucose tolerance, lipid profile, energy balance, appetite, and temperature[11]. These facts not only emphasize the sophistication of human circadian mechanisms, but also the relevance of further exploration to fully understand their implications on health and disease.

2.1. Cardiovascular system

The pulsatile nature of cardiac and vascular functions is clearly governed by circadian rhythms. They ensure the adaptation to the cyclic demands of human body, optimizing physiological functions during periods of wakefulness and activity, and conserving energy during rest. Excellent examples are heart rate and blood pressure; they do not remain static but fluctuate significantly over the 24-hour period. Heart rate daily variations have been attributed to central neurohumorally mediated circadian cues[12] and the cardiomyocyte circadian clock[13]. Blood pressure raises prior to awakening, peaks in the mid-morning, and decreases towards sunset[12]. Such pattern is linked to the intrinsic properties of blood vessels, not merely due to activity or rest but also regulated by the molecular clocks[10].

Many other cardiovascular aspects are temporally modulated: endothelial function, thrombus formation, cardiac metabolism, responsiveness to extracellular signals, contractility, signaling, and even heart's growth and regeneration processes[14]. Of note, acute cardiovascular events (e.g., myocardial infarction, stroke, or arrhythmias) occur more frequently in the morning, which illustrate further the circadian dynamics in cardiovascular health[12].

2.2. Glycemic metabolism

The circadian regulation of glycemic metabolism is robustly documented. Under standard light-dark cycles, glucose levels peak at waking and dip during sleep in healthy humans. Indicating a fundamental circadian mechanism, the rhythm persists independent of dietary intake and fasting at different times[16].

In the late 1960's and 1970's, oral glucose tolerance was the first evidence for circadian regulation of glycemic metabolism[17]. With a large magnitude of variation, oral glucose tolerance typically exerts a morning zenith and gradually declines into the evening [11]. Individuals who present normal glucose tolerance in the morning show similar values to those with prediabetes in the evening[18]. This striking variation can be explained by heightened β-cell activity and insulin sensitivity in the morning compared to later times of the day[11].

Peripheral glucose uptake mediated by both core intracellular pathways and circulating factors contribute to the circadian variation in glycemic control. Within the skeletal muscle, approximately 15% of transcripts demonstrate a rhythmic expression pattern with particular emphasis on genes involved in glucose and lipid metabolism[19]. Glycogen stores

peak in the evening both in muscle and the liver[20], while insulin sensitivity declines as the sun sets[21]. Regarding circulating factors, the central clock in the SCN regulates growth hormone[22] and glucocorticoid secretion[23]. Growth hormone – an agent of insulin resistance[24] – increase shortly after sleep onset and decrease before waking up[25]. Glucocorticoid release – which promote hyperglycemia – peaks in the early morning regulated by both the SCN through the sympathetic nervous system[23] and the peripheral clock in the adrenal gland[26].

The circadian regulation of glycemic metabolism ensures optimal energy utilization throughout the day. During sleep, endogenous glucose production boost, peaking at dawn to prepare for the day's activities. Then, it gradually declines as food intake provides external glucose sources. Likewise, the cycle aligns the natural light-dark cycle with hormonal responses to maintain euglycemia and facilitate the transition from fasting/eating and rest/activity states.

2.3. Lipid metabolism

Lipids exhibit more extensive circadian regulation than any other plasma metabolites[27]. However, rhythmicity in lipids is not completely understood at present. Cholesterol (both total and LDL) and triglycerides show modest to large circadian rhythms with a plurality of acrophases peaking in the morning or around noon. Meanwhile, the evidence for a diurnal rhythm in HDL-C remains inconclusive. Discrepancies in lipids circadian regulation has been found between distinct populations and between sexes[11]. Therefore, the existence of distinct metabolic phenotypes has been suggested [28].

Lipid transport in mitochondria –the central organelle in cellular energy production – shows circadian patterns. Acylcarnitines, which are essential in fatty acid transport to mitochondria for lipid oxidation, also display diurnal rhythms[29]. It has been observed that mitochondrial fat oxidation in the skeletal muscle peaks around 23:00 h in healthy men[19]. Reflecting potential sexual dimorphism, a shift from morning fatty acid oxidation to evening lipogenesis has been seen in in overweight, healthy women[30]. Further, fat oxidation during a single bout of exercise at maximal fat oxidation intensity is higher in the evening vs. the morning in men[31], but not women[32]. The available knowledge highlights the complexity and still underexplored circadian regulation of lipid metabolism.

2.4. Energy metabolism

The SCN communicates with several hypothalamic and extra-hypothalamic areas (i.e., the supraventricular region, paraventricular nucleus, arcuate nucleus, lateral geniculate nucleus, and raphe nuclei) responsible for energy balance regulation (which includes energy expenditure and energy intake) and temperature[33].

Daily energy expenditure and intake is tightly adjusted to maintain energy balance. When catabolic neuronal pathways – those that reduce food intake and increase energy expenditure – are activated, anabolic pathways – those that stimulate food intake and decrease energy expenditure – are strongly inhibited[34]. The anticipatory nature of the circadian system naturally promotes fasting during the night and food intake during the wake period[35]. The orexigenic hormone ghrelin shows highest levels in the afternoon and gradually declines during the night[36]. Additionally, self-reported hunger peaks in the evening[11]. Theoretically, consuming larger meals prior to the sleep would support the consequent fasting period and mitigate any increase in hunger that could potentially disrupt the resting phase[35]. In coherence, the anorexigenic hormone leptin peaks during sleep[37] and fat oxidation may be primed at this time to support the fasting state[38]. Energy expenditure manifest maximal values in the biological morning, predominantly influenced by its postprandial component. The thermic effect of food can be up to 44% higher in the morning than in the evening which suggest optimized catabolic processes at this time-of-day[39].

Although partly driven by central molecular clocks, energy balance is not independent of external cues. For example, food acts as a strong zeitgeber capable of entraining peripheral clocks[40]. Indeed, circadian fluctuations in leptin and ghrelin are importantly influenced by habitual meal timing and sleep patterns[41].

Lastly, body temperature exhibits peak values in the late afternoon and early evening, while dip during the early morning hours. The thermal pattern is intimately related to sleep that typically occurs as body temperature begins its nightly decline. Efficient transitions between wakefulness and sleep result essential for optimal energy use and physiological recovery[42].

Given these insights, the interplay between molecular clocks and cardio-metabolic processes becomes not merely a physiological phenomenon, but a foundation of human's health. Deeper understanding of the circadian physiology potentially holds the key to

improved public health guides, new therapeutic strategies and tailored interventions to align more closely with our natural biological rhythm.

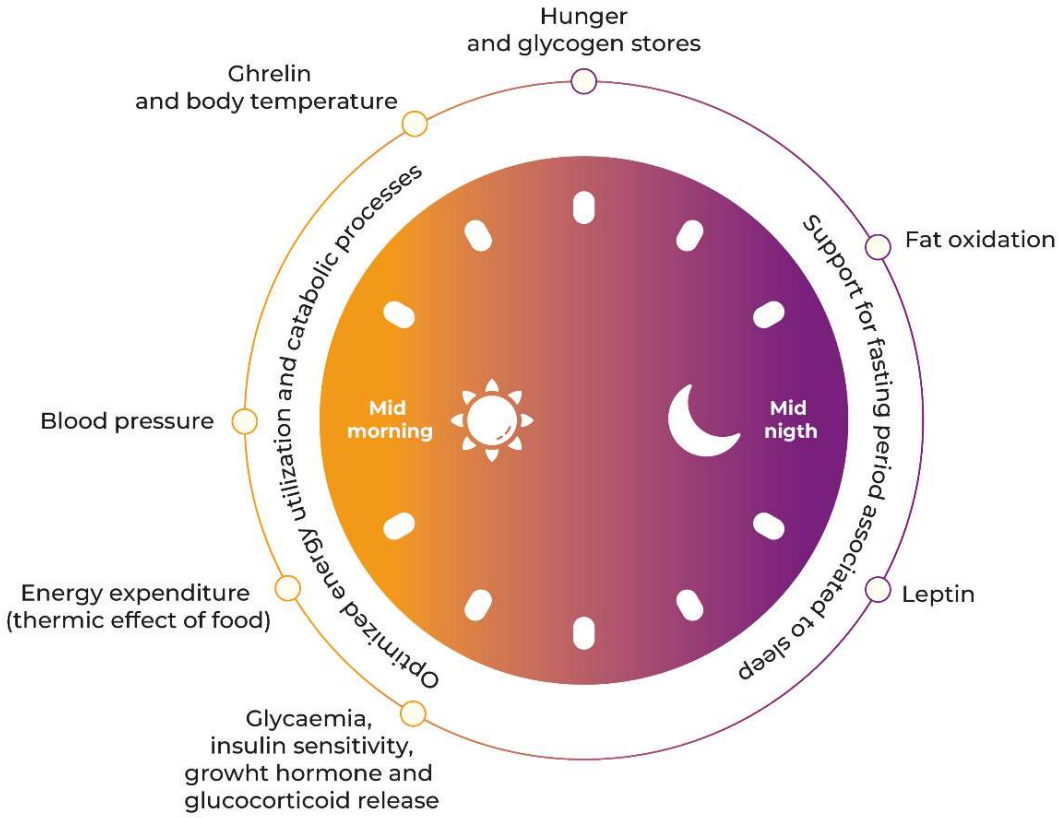


Figure 4. Circadian rhythmicity of cardiometabolic parameters. Small white circles represent approximately the time-of-day when levels of each parameter are higher. Glucocorticoid and growth hormone release peak in the early morning, leading to higher glycemia at this time-of-day, and insulin sensitivity is also enhanced[24,25]. Energy expenditure is higher in the morning, primarily due to the thermic effect of food[39]. Blood pressure peaks in the mid-morning[12]. In the afternoon and early evening, body temperature and ghrelin levels are at their highest[36,43]. Perceived hunger peaks in the evening[11]. Glycogen stores in the muscle and liver are replenished during the evening[20,21]. Mitochondrial fat oxidation peaks before midnight [19]. During sleep, leptin levels are at their highest, along with optimized fat oxidation[37,38]. Overall, energy utilization and catabolic processes are promoted in the morning, while in the evening, energy stores are replenished, and metabolic processes are optimized to support the fasting period associated with sleep.

3. Circadian misalignment is detrimental for health

It has already been mentioned that circadian rhythms at the whole-body level are regulated not only by the cell's intrinsic molecular clocks but also by zeitgebers such as light-dark exposure, sleep, food intake, or physical activity. Growing evidence indicates that when these external cues become misaligned with the endogenous circadian systems (e.g., through exposure to bright light at night, insufficient or shifted sleeping, or nocturnal eating) multiple health-related aspects result adversely affected.

Studies conducted in both animals and humans attribute the disruptions of circadian rhythms to the development of numerous diseases. In rodents, mutations in molecular clock genes have demonstrated to cause cardiac arrhythmias[19], atherosclerosis, diminished blood pres/sure rhythmicity, insulin resistance or altered oxygen consumption and fatty acid oxidation[10]. Research from rodent models also indicates that caloric intake during periods typically reserved for sleep leads to greater weight gain compared to consuming the same amount of calories during wakeful periods[19]. While complete dysfunction of clock genes (often used in animal models) is rare in humans, genome-wide association studies have found several clock gene single nucleotide polymorphisms robustly associated with hypertension, diabetes and metabolic syndrome[44]. Moreover, observational studies in humans demonstrate a clear relationship between circadian disruption and cardiometabolic disease[45] .

Much research about circadian misalignment in humans has been performed in shift workers. Shift work, a pattern which disrupts normal sleep-wake cycles and requires individuals to be active at night, serves as a prime example of how circadian misalignment affects health. These individuals face significantly higher systolic and diastolic blood pressure levels and an increased risk of cardiovascular disease[10], diabetes and metabolic syndrome[45]. Shift work is also linked to a poorer lipid profile and a higher likelihood of presenting overweight or obesity [45]. Additionally, irregular or insufficient sleep themselves are associated to cardiovascular disease, hypertension, type 2 diabetes, dyslipidemia and obesity[41].

The mechanisms underlying these associations are not fully understood. Observational studies are limited in this context because of the lack of direct measurements and the absence of causality. Potential mechanisms include increased inflammation[45], impaired regulation of blood pressure, and decreased production of vasoactive substances essential for vascular health[10]. Additionally, eating at the "wrong" time, when glucose tolerance is

reduced, could cause desynchronization between clocks in diverse tissues [3]. Both circadian misalignment and disrupted sleep patterns can affect 24-hour energy expenditure and modify appetite hormones, thereby promoting hunger and energy intake. Of note, total energy intake among shift workers does not appear to exceed that of non-shift workers; however, their dietary choices tend to be less healthy [41].

In summary, sleep and circadian rhythms are becoming evident pillars for optimal health, as well as nutrition and exercise, in contemporary society.

4. Exercise is a zeitgeber with the potential to optimize cardiometabolic health

A big amount of evidence supports exercise as a crucial preventative and therapeutic method for improving cardiometabolic health. With this 'simple' non-invasive therapy, we can obtain improvements in blood pressure, insulin sensitivity and glucose tolerance, lipid profile, energy metabolism and appetite[46,47].

4.1. Cardiometabolic adaptations to exercise

The health-related benefits of exercise stem from a cascade of physiological adaptations in response to increased energy demands. On the basis, an increase in physical activity requires changes in the distribution of oxygen and nutrients through the body to ultimately meet the rising demands of ATP in the skeletal muscle. During endurance exercise – which is high oxygen demander – ventilation and cardiac output increase and vascular resistance decreases. Vasodilatation, hyperthermia, and increased blood flow also contribute to facilitate the use of oxygen in the skeletal muscle. In contrast, during resistance training – which mainly activate anaerobic metabolic pathways –, blood pressure increases depending on the intensity of effort and muscle mass, with cardiac output increasing only modestly[48]. The vasodilatory response makes moderate-intensity aerobic exercise to induce a transient reduction in blood pressure in both normotensive and hypertensive individuals[49]. Additionally, it produces a decreased vascular resistance and blood viscosity, adaptations that promote an antithrombotic physiological status. The elevated energy demand caused by exercise further contributes to cardiovascular health by preventing or alleviating plasma dyslipidemia and reducing high plasma triglyceride concentrations[47].

In the skeletal muscle, free fatty acids mobilized from the adipose tissue are an important source of energy during low to moderate intensity prolonged exercise. When engaging in long-term vigorous exercise, training adaptations occur in muscle cells to enhance aerobic

work capacity. These include increased number of mitochondria and respiratory enzyme activity, both improving muscle's ability to utilize oxygen more efficiently[50]. With increasing exercise intensity, carbohydrates become a mounting important energy substrate[51]. Specifically, at intensities around 85% maximum oxygen uptake (VO_2max) or higher, carbohydrate oxidation accounts from 70% to 100% of energy consumption[52]. The active muscles obtain glucose molecules coming from plasma glucose and muscle glycogen-breakdown, being the latter the predominant source at the highest intensities[51]. During exercise, translocation of glucose transporter type 4 (GLUT4) from the muscle cell's cytoplasm to the membrane makes possible the enhanced permeability of this molecule[53]. After exercise, in need to rebuild glycogen stores, muscle glucose uptake displays an increased sensitivity to insulin[51]. These adaptations become stable with regular training enabling the body to use glucose more efficiently and reducing the risk of cardiometabolic diseases such as type 2 diabetes[46].

Lastly, exercise is acknowledged as one of the most valuable components of behavior that can influence body weight helping in the prevention and management of obesity. Exercise increases energy expenditure thus modifies energy balance, potentially generating an energy deficit. The extra energy expenditure, however, also has an impact on appetite and energy intake[54]. First theories about energy balance proposed that an increase in energy output due to exercise should be automatically followed by an equivalent increase in caloric intake[55]. After, it was postulated that energy expenditure and intake are linked in a J-shaped curve where energy balance is achieved only under conditions of high energy expenditure. Contrary, energy expenditure and intake become unmatched under sedentary conditions with poor homeostatic control over appetite, consequent overconsumption[56], and even preference for high-energy food[57]. The match between energy expenditure and energy balance is promoted by exercise via its effects on both 'episodic' – satiety cues consequence of food consumption – and 'tonic' – cues from body tissues and metabolism – signals[54]. Previous research consistently shows that acute responses to exercise (i.e., aerobic session above the 60% VO2max) include changes in gastrointestinal hormones such as the suppression of ghrelin and the release of anorexigenic peptides (i.e., glucagon-like peptide-1 [GLP-1] and peptite YY [PYY])[58]. In addition, long-term training is frequently accompanied by an increase of fat free mass and loss of fat mass. Higher fat free mass supposes higher demands of energy and translate into enhanced basal hunger; interestingly, it is accompanied by increased postprandial

satiety and better meal-to-meal appetite control, both potentially explained by the fat mass loss and the improved insulin sensitivity[54].

4.2. Myokines: the endocrine role of the skeletal muscle

At the molecular level, exercise leads to the production of certain signaling molecules – the so called 'myokines' – that importantly mediate metabolic adaptations[59]. In this context, the skeletal muscle functions as an endocrine structure producing and releasing myokines that influence metabolism of remote organs[60].

One of the first signaler studied as a myokine was muscle-derived interleukin 6 (IL-6). IL-6 was proposed as a 'true' exercise factor after acknowledging that its encoding gene is silent in the skeletal muscle in resting conditions, but rapidly activated by contractions[61]. Serving as an energy sensor, IL-6 levels are enhanced especially if muscle glycogen levels are depleted. Following exercise, IL-6 is released into the circulation to mediate some of the exercise effects in other organs and tissues, such as the liver or adipose tissue. In humans, IL-6 myokine increases insulin-stimulated glucose disposal and promotes lipolysis and fatty acid oxidation[62].

FGF21 and GDF15 are other pivotal myokines in adjusting human energy homeostasis in response to exercise. Particularly, they are critically involved in lipids metabolism. FGF21 is upregulated in the skeletal muscle under energy deficit conditions and other physiological stresses. It facilitates fatty acids oxidation and promotes the mobilization of systemic fat stores. Similarly, GDF15 is induced in the skeletal muscle upon contraction and, regulated in a homeostatic loop, increases the mobilization of fat stores in the adipose tissue[63]. In this manner, myokines partly mediate the protection against cardiometabolic disease attributed to regular exercise.

As energy regulatory endocrine signals, myokines are additionally involved in the exercise-induced changes on appetite and food intake. IL-6 contributes to exercise-induced appetite suppression by stimulating the secretion of GLP-1 and PYY from intestinal L cells [64]. On the other hand, lactate binds gastric cells and inhibit the secretion of acylated ghrelin[64]. Both IL-6 and lactate are released in an intensity-dependent manner and have demonstrated to reduce energy intake in humans[64,65]. Greater changes in lactate and IL-6 after acute exercise lead to greater changes in the circulating gut hormones[66]. Interestingly, after intense exercise the enzyme CNDP2 transforms lactate to lactate-phenylalanine (Lac-Phe)[67]. Novel insights position Lac-Phe as a promising molecule able to reduce food intake, plausibly via a dual role via central nervous system and adipose

tissue[68]. Also, GDF15 has recently gained special attention as a promising anti-obesity therapy. Late data show that GDF15 travels through the bloodstream, reaches the brainstem, and triggers a reduction in food consumption, among other hormonal and behavioral reactions[69]. At the moment of exercise and particularly when it is of high intensity, mechanisms to avoid eating could help to maintain blood flow in the skeletal muscle [68].

4.3. Skeletal muscle and the molecular clock

As illustrated in Figure 3 in the present chapter, almost every cell in the body possesses its own core clock machinery. Skeletal muscle cells are not an exception; they have an extensive network of clock-controlled genes able to oscillate even without interfering with the SCN, light-dark cues or behavior. In the skeletal muscle, the activity of the core clock machinery seems to be closely aligned to metabolic flux[70]. For example, core clock genes (specifically, *CRY1* and *PER2*) can respond to modulation of a key energy sensor in the skeletal muscle (i.e., AMPK)[71]. Also, the amplitude of *NR1D1* gene expression (coding *Rev-erb-α*) correlates with insulin sensitivity and is associated with training status[72]. Another parameter relating the skeletal muscle clock and metabolism is mitochondrial function. Mitochondrial dynamics oscillate in a circadian manner, involving the fusion and fission of mitochondria, which is crucial process for adapting cell homeostasis to energy demands[73]. Both acute and chronic exercise potently remodels mitochondrial dynamics, and simultaneously the core clock in skeletal muscle can respond to alteration in these dynamics. Presumably, aligning exercise sessions with times of active mitochondrial dynamics could suppose an improvement in substrate uptake and utilization or intensify the remodeling of the mitochondrial network[15].

The interaction between the time-of-day and exercise performance is extensively studied. Higher exercise intensity correlates with greater exercise capacity in the afternoon/evening. Ameliorated neuromuscular performance, increased temperature and better ability to dissipate body heat, among others, are factors probably contributing to this phenomenon[15].

Less research exists regarding diurnal timing of exercise in terms of cardiometabolic health outcomes, although accumulating evidence from rodent models suggest that the molecular clock in the skeletal muscle directly modulate the exercise response[74,75].

4.4. Exercise as a zeitgeber

Exercise – and particularly the one of higher intensity – is a robust zeitgeber of skeletal muscle clocks[15]. A better understanding of the interaction between time-of-day of exercise and health-related parameters becomes important to potentially ameliorate the negative cardiovascular and metabolic effects linked to circadian misalignment.

Recent epidemiological studies manifest the significance of the time of exercise in terms of its cardiometabolic effects. In a large cohort of 86,657 individuals, morning physical activity measured through triaxial accelerometers were associated with a lower risk of developing cardiovascular diseases[76]. Conversely, engaging in moderate-to-vigorous physical activity in the afternoon or evening has shown to reduce insulin resistance by up to 25% in participants of the "Netherlands Epidemiology of Obesity Study"[77]. Data from the "Active and Healthy Ageing" study – which recruited 207 individuals and utilized wrist-worn accelerometers for assessing physical activity – demonstrated that timing affects metabolic parameters differently throughout the day[78]. Specifically, early morning activities were linked to lower fasting glucose and insulin levels, whereas late afternoon activities were associated with lower BMI. Notably, activities conducted at night were correlated with higher BMI and fasting glucose levels.

Research on the impact of the time-of-day of exercise extends beyond observational to interventional studies. A specific intervention for shift-workers served as a proof of concept to demonstrate the importance of correctly scheduling the time of exercise for a given population. After participants cycling for 15 minutes every hour during eight consecutive night shifts, there was a phase delay in temperature rhythm that aligned better with daytime sleep[79].

Several works suggest that evening exercises might help prevent hyperglycemic events in patients with type 2 diabetes[80–83]. Meanwhile, very recent findings indicate that morning high-intensity interval exercise compared to afternoon significantly reduce systolic blood pressure and insulin levels, though not fasting glucose, in individuals with metabolic syndrome[84]. Further, moderate- to high-intensity aerobic exercise performed in the morning has been identified as potentially more effective than evening exercise for controlling appetite, reducing calorie intake, and facilitating weight loss in inactive overweight women[85]. Interestingly, a recent study reported opposite results when studying men and women. A 12-week multimodal exercise intervention resulted in reduced abdominal fat and blood pressure after morning exercise in women while, for

men, evening exercise increased fat oxidation and reduced systolic blood pressure and fatigue[86].

Collectively, these findings remark the existence of diurnal variations in cardiometabolic outcomes after exercise, with evident heterogeneity across interventions and populations. It is particularly of emphasis that the optimal timing of exercise might differ not just based on lifestyle or health condition, but also on sex. Increasing research into how the time-of-day of exercise impacts cardiometabolic health is imperative to target specific populations ultimately optimizing the power of exercise as a preventative and therapeutic tool.

5. Biological sex differences must be taken into consideration in exercise research

It is undeniable that biological sex significantly influences exercise physiology. Throughout this document, we use the term 'sex' to refer to the biological differences between males and females, including variations in chromosomes and profiles of endogenous sex hormones. We also refer to males and females as men and women, respectively.

Biological sex is a primary determinant of athletic performance, being the primary explanation the higher endogenous testosterone levels in men[87]. Testosterone exerts powerful anabolic effects on muscle and bone mass, greater of those exerted by the main sex steroid hormone in women (i.e., estradiol). Fundamental sex hormones differences lead to men generally being taller and heavier, with higher lean body mass and a lower percentage of fat mass compared to women. Men possess larger, stronger, and faster skeletal muscles with predominantly increased amount of type II fibers. Conversely, women have a higher proportion of type I fibers what gives them higher rates of relaxation and a more fatigue-resistant muscle. Additionally, endogenous testosterone levels are related to higher circulating hemoglobin and larger heart size, leading to a higher VO2max and greater aerobic capacity in men compared to women. Partly due to differences in muscle fiber types, women exhibit a greater oxidative capacity, while men demonstrate a greater glycolytic capacity. During endurance exercise at equal intensity, women tend to oxidize more fat and fewer carbohydrates and amino acids than men[88].

In women, hormonal fluctuations across the menstrual cycle can impact exercise performance. Estradiol is associated with increases in free fatty acid utilization, glycogen conservation, and muscle glycogen storage[87,89]. However, a recent large meta-analysis shows that these effects are minimal in terms of overall sports performance[90].

Crucially, key questions on the influence of the menstrual cycle on exercise physiology remain underexplored because the current literature is mostly based on men. Cowan and colleagues found that only 10% of participants from original studies in major sport and exercise medicine/physiotherapy journals were women, a statistic that had not changed significantly over a 10-year period until 2019[91]. Frequently, the potential effect of the menstrual cycle has been used to exclude female from studies' samples. Argument that, however, is no longer accepted. Female physiological parameter can be confounding variables as much as others (e.g., temperature, nutrition, fatigue, etc.) that are routinely well handled in most studies[92].

Future directions in exercise science emphasize the necessity of including sex as a biological variable across all studies. This approach would involve the inclusion of women at all levels of research (which also includes authorship)[93], ensuring studies to be adequately powered to understand sex differences in the acute and chronic response to exercise. Only in this way it will be possible to create evidence-based recommendations for training individualization also in women[88].

6. Gaps and directions

The present chapter highlights the intricate relationship between the molecular clock and cardiometabolic health. As cardiometabolic diseases continue to rise in prevalence within our society, exploring ways to optimally prevent and treat these conditions becomes essential. Exercise is as a well-recognized ally for maintaining a proper overall health. Yet, the link between time-of-day and exercise has been explored more from the standpoint of performance than a health perspective. Importantly, women remain underexplored in exercise research, a disparity that must be addressed, especially in this field where some studies suggest the existence of sexual dimorphism. Understanding the interplay between molecular clocks and exercise will optimize exercise as a therapeutic tool, conducting to more precise exercise prescription in the clinical settings

7. References

1. Panda S, Hogenesch JB, Kay SA. Circadian rhythms from flies to human. Nature 2002; 417:329–335.

2. Mohawk JA, Green CB, Takahashi JS. Central and peripheral circadian clocks in mammals. Annu Rev Neurosci 2012; 35:445.

3. Panda S. Circadian physiology of metabolism. Science (1979) 2016; 354(6315):1008-1015.

4. Koronowski KB, Sassone-Corsi P. Communicating clocks shape circadian homeostasis. Science (1979) 2021; 371(6530).

5. Takahashi JS, Hong HK, Ko CH, McDearmon EL. The genetics of mammalian circadian order and disorder: Implications for physiology and disease. Nat Rev Genet 2008; 9(10):764-775.

6. Dibner C, Schibler U, Albrecht U. The Mammalian Circadian Timing System: Organization and Coordination of Central and Peripheral Clocks. Annu Rev Physiol 2010; 72(1):517-549.

7. Welsh DK, Logothetis DE, Meister M, Reppert SM. Individual Neurons Dissociated from Rat Suprachiasmatic Nucleus Express Independently Phased Circadian Firing Rhythms. Neuron 1995; 14:697-706.

8. Panda S, Provencio I, Tu DC, et al. Melanopsin Is Required for Non-Image-Forming Photic Responses in Blind Mice. Curr Opin Neurobiol 1997; 78(23):1.

9. Ishida A, Mutoh T, Ueyama T, et al. Light activates the adrenal gland: Timing of gene expression and glucocorticoid release. Cell Metab 2005; (5):297-307.

10. Crnko S, Du Pré B, Sluijter JPG, Van Laake LW. Circadian rhythms and the molecular clock in cardiovascular biology and disease. Nat Rev Cardiol 2019; 16(7):437-447.

11. Poggiogalle E, Jamshed H, Peterson CM. Circadian Regulation of Glucose, Lipid, and Energy Metabolism in Humans. Mol Metab 2018; 84:11-27.

12. Millar-Craig MW, Bishop CN, Raftery EB. Circadian variation of blood-pressure. The Lancet 1978; 311(8068):795-797.

13. Bray MS, Shaw CA, Moore MWS, et al. Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression. Am J Physiol Heart Circ Physiol 2008; 294(2):1036-1047.

14. Durgan DJ, Young ME. The Cardiomyocyte Circadian Clock: Emerging Roles in Health and Disease. Circ Res 2010; 106(4):647–658.

15. Gabriel BM, Zierath JR. Circadian rhythms and exercise - re-setting the clock in metabolic disease. Nat Rev Endocrinol 2019; 15(4):197-206.

16. Peng F, Li X, Xiao F, Zhao R, Sun Z. Circadian clock, diurnal glucose metabolic rhythm, and dawn phenomenon HHS Public Access. Trends Neurosci 2022; 45(6):471-482.

17. Jarrett RJ, Keen H. Diurnal variation of oral glucose tolerance: a possible pointer to the evolution of diabetes mellitus. Br Med J 1969; 2(5653):341-344.

18. Carroll KF, Nestel PJ. Diurnal variation in glucose tolerance and in insulin secretion in man. Diabetes 1973; 22(5):333-348.

19. Van Moorsel D, Hansen J, Havekes B, et al. Demonstration of a day-night rhythm in human skeletal muscle oxidative capacity 2016; 5(8):635-645.

20. Macauley M, Smith FE, Thelwall PE, Hollingsworth KG, Taylor R. Diurnal variation in skeletal muscle and liver glycogen in humans with normal health and Type 2 diabetes. Clin Sci 2015; 128(10):707-713.

21. Gibson T, Jarrett RJ. Diurnal variation in insulin sensitivity. The Lancet 1972; 300(7784):947-948.

22. Vakili H, Jin Y, Cattini PA. Evidence for a Circadian Effect on the Reduction of Human Growth Hormone Gene Expression in Response to Excess Caloric Intake. J Biol Chem 2016; 291(26):13823-33.

23. Buijs RM, Wortel J, Heerikhuize JJ Van, et al. Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. European Journal of Neuroscience 1999; 11(5):1535-1544.

24. Møller N, Jørgensen JO, Abildgård N, Orskov L, Schmitz O, Christiansen JS. Effects of growth hormone on glucose metabolism. Horm Res 1991; 36(1):32-35.

25. Van Cauter E, Blackman JD, Roland D, Spire JP, Refetoff S, Polonsky KS. Modulation of Glucose Regulation and Insulin Secretion by Circadian Rhythmicity and Sleep. J Clin Invest 1991; 88(3):934-942.

26. Oster H, Challet E, Ott V, et al. The Functional and Clinical Significance of the 24-Hour Rhythm of Circulating Glucocorticoids. Endocr Rev 2017; 38(1):3.

27. Dallmann R, Viola AU, Tarokh L, Cajochen C, Brown SA. The human circadian metabolome. Proc Natl Acad Sci U S A 2012; 109(7):2625-2629.

28. Chern-Pin Chua E, Shui G, Lee ITG, et al. Extensive diversity in circadian regulation of plasma lipids and evidence for different circadian metabolic phenotypes in humans. Proc Natl Acad Sci U S A 2013; 110(35):14468-73.

29. Ang JE, Revell V, Mann A, et al. Identification of Human Plasma Metabolites Exhibiting Time-of-Day Variation Using an Untargeted Liquid Chromatography-Mass Spectrometry Metabolomic Approach. Chronobiol Int 2012; 29(7):868-881.

30. Yoshino J, Almeda-Valdes P, Patterson BW, et al. Diurnal Variation in Insulin Sensitivity of Glucose Metabolism Is Associated With Diurnal Variations in Whole-Body and Cellular Fatty Acid Metabolism in Metabolically Normal Women. J Clin Endocrinol Metab 2014; 99(9):E1666-E1670.

31. Amaro-Gahete FJ, Jurado-Fasoli L, Triviño AR, et al. Diurnal Variation of Maximal Fat-Oxidation Rate in Trained Male Athletes. Int J Sports Physiol Perform 2019; 14(8):1140-1146.

32. Robles-González L, Aguilar-Navarro M, López-Samanes Á, et al. No diurnal variation is present in maximal fat oxidation during exercise in young healthy women: A cross-over study. Eur J Sport Sci 2023; 23(6):936-942.

33. Page AJ, Christie S, Symonds E, Li H. Circadian regulation of appetite and time restricted feeding. Physiol Behav 2020; 220:112873.

34. Schwartz MW, Woods SC, Seeley RJ, Barsh GS, Baskin DG, Leibel RL. Is the Energy Homeostasis System Inherently Biased Toward Weight Gain? Diabetes 2003; 52:232-238.

35. Scheer FAJL, Morris CJ, Shea SA. The internal circadian clock increases hunger and appetite in the evening independent of food intake and other behaviors. Obesity 2013; 21(3):421-423.

36. Natalucci G, Riedl S, Gleiss A, Zidek T, Frisch H. Spontaneous 24-h ghrelin secretion pattern in fasting subjects: maintenance of a meal-related pattern. Eur J Endocrinol 2005; 152(6):845-850.

37. Sinha MK, Ohannesian JP, Heiman ML, et al. Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. Journal of Clinical Investigation 1996; 97(5):1344.

38. Rynders CA, Morton SJ, Bessesen DH, Wright KP, Broussard JL. Circadian Rhythm of Substrate Oxidation and Hormonal Regulators of Energy Balance. Obesity 2020; 28(S1):S104-S113.

39. Morris CJ, Garcia JI, Myers S, Yang JN, Trienekens N, Scheer FAJL. The human circadian system has a dominating role in causing the morning/evening difference in early diet-induced thermogenesis. Obesity (Silver Spring) 2015; 23(10):2053.

40. Zilberter T, Dumbell RA, Helfer G, Sung HK, Pickel L. Feeding Rhythms and the Circadian Regulation of Metabolism. Frontiers in Nutrition 2020; 1:39.

41. Chaput JP, McHill AW, Cox RC, et al. The role of insufficient sleep and circadian misalignment in obesity. Nat Rev Endocrinol 2023; 19(2):82-97.

42. Kräuchi K. The thermophysiological cascade leading to sleep initiation in relation to phase of entrainment. Sleep Med Rev 2007; 11(6):439-451.

43. Kräuchi K, Cajochen C, Wirz-Justice A. Thermophysiologic aspects of the three-process-model of sleepiness regulation. Clin Sports Med 2005; 24(2):287-300.

44. Anafi RC, Francey LJ, Hogenesch JB, Kim J. CYCLOPS reveals human transcriptional rhythms in health and disease 2017; 114.

45. Ansu Baidoo V, Knutson KL. Associations between circadian disruption and cardiometabolic disease risk: A review. Obesity 2023; 31(3):615-624.

46. Gabriel BM, Zierath JR. The Limits of Exercise Physiology: From Performance to Health. Cell Metab 2017; 25(5):1000-1011.

47. Tian D, Meng J. Exercise for prevention and relief of cardiovascular disease: Prognoses, mechanisms, and approaches. Oxid Med Cell Longev 2019; 2019:3756750.

48. Fulghum K, Hill BG. Metabolic Mechanisms of Exercise-Induced Cardiac Remodeling. Front Cardiovasc Med 2018; 5:127.

49. Pescatello LS, Kulikowich JM. The Aftereffects of Dynamic Exercise on Ambulatory Blood Pressure 2001; 33(11):1855-61.

50. Holloszy J 0. Biochemical adaptations in muscle effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. Journal of Biological Chemistry 1967; 242(9):2278-2282.

51. Jensen TE, Richter EA. Regulation of glucose and glycogen metabolism during and after exercise. Journal of Physiology 2012; 590(5):1069-1076.

52. Romijn JA, Coyle EF, Sidossis LS, et al. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration 1993; 265(3 Pt 1):E380-91.

53. Lund S, Holmant GD. Contraction Stimulates Translocation of Glucose Transporter GLUT4 in Skeletal Muscle through a Mechanism Distinct from That of Insulin. Proc Natl Acad Sci U S A 1995; 92(13):5817-21.

54. Blundell JE, Gibbons C, Caudwell P, Finlayson G, Hopkins M. Appetite control and energy balance: impact of exercise. Obesity Reviews 2015; 16(S1):67-76.

55. Mayer J, Roy P, Mitra KP. Relation between Caloric Intake, Body Weight, and Physical Work. Am J Clin Nutr 1956; 4(2):169-175.

56. Blundell JE. Physical activity and appetite control: can we close the energy gap? Nutr Bull 2011; 36(3):356-366.

57. Beaulieu K, Oustric P, Finlayson G. The Impact of Physical Activity on Food Reward: Review and Conceptual Synthesis of Evidence from Observational, Acute, and Chronic Exercise Training Studies. Curr Obes Rep 2020; 9(2):63-80.

58. Thackray AE, Stensel DJ. The impact of acute exercise on appetite control: Current insights and future perspectives. Appetite 2023; 186:106557.

59. Karstoft K, Pedersen BK. Skeletal muscle as a gene regulatory endocrine organ. Curr Opin Clin Nutr Metab Care 2016; 19(4):270-275.

60. Trayhurn P, Drevon CA, Eckel J. Secreted proteins from adipose tissue and skeletal muscle – adipokines, myokines and adipose/muscle cross-talk. Arch Physiol Biochem 2011; 117(2):47-56.

61. Febbraio MA, Pedersen BK. Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. FASEB J 2002; 16(11):1335-1347.

62. Pedersen BK, Fischer CP. Beneficial health effects of exercise – the role of IL-6 as a myokine. Trends Pharmacol Sci 2007; 28(4):152-156.

63. Jena J, García-Peña LM, Pereira RO. The roles of FGF21 and GDF15 in mediating the mitochondrial integrated stress response. Front Endocrinol (Lausanne) 2023; 14:1264530.

64. Almada C, Cataldo LR, Smalley S V., et al. Plasma levels of interleukin-6 and interleukin-18 after an acute physical exercise: relation with post-exercise energy intake in twins. J Physiol Biochem 2013; 69(1):85-95.

65. Schultes B, Schmid SM, Wilms B, Jauch-Chara K, Oltmanns KM, Hallschmid M. Lactate infusion during euglycemia but not hypoglycemia reduces subsequent food intake in healthy men. Appetite 2012; 58(3):818-821.

66. Islam H, Townsend LK, McKie GL, Medeiros PJ, Gurd BJ, Hazell TJ. Potential involvement of lactate and interleukin-6 in the appetite-regulatory hormonal response to an acute exercise bout. J Appl Physiol 2017; 123(3):614-623.

67. Li VL, He Y, Contrepois K, et al. An exercise-inducible metabolite that suppresses feeding and obesity. Nature 2022 606:7915 2022; 606(7915):785-790.

68. Caruso L, Zauli E, Vaccarezza M. Physical Exercise and Appetite Regulation: New Insights. Biomolecules 2023; 13(8):1170.

69. Klein AB, Kleinert M, Richter EA, Clemmensen C. GDF15 in Appetite and Exercise: Essential Player or Coincidental Bystander? Endocrinology 2022; 163(1):bqab242.

70. Perrin L, Loizides-Mangold U, Chanon S, et al. Transcriptomic analyses reveal rhythmic and CLOCK-driven pathways in human skeletal muscle. Elife 2018; 7:e34114.

71. Wolf G, Esser KA. Scheduled Exercise Phase Shifts the Circadian Clock in Skeletal Muscle. Med Sci Sports Exerc 2012; 44(9):1663-1670.

72. Chtourou H, Souissi N. The effect of training at a specific time of day: A review. J Strength Cond Res 2012; 26(7):1984-2005.

73. van Moorsel D, Hansen J, Havekes B, et al. Demonstration of a day-night rhythm in human skeletal muscle oxidative capacity. Mol Metab 2016; 5(8):635-645.

74. Jordan SD, Kriebs A, Vaughan M, et al. CRY1/2 Selectively Repress PPARδ and Limit Exercise Capacity. Cell Metab 2017; 26(1):243-255.e6.

75. Peek CB, Levine DC, Cedernaes J, et al. Circadian Clock Interaction with HIF1α Mediates Oxygenic Metabolism and Anaerobic Glycolysis in Skeletal Muscle. Cell Metab 2017; 25(1):86-92.

76. Albalak G, Stijntjes M, Van Bodegom D, et al. Setting your clock: associations between timing of objective physical activity and cardiovascular disease risk in the general population. Eur J Prev Cardiol 2022; 00:1-9.

77. van der Velde JHPM, Boone SC, Winters-van Eekelen E, et al. Timing of physical activity in relation to liver fat content and insulin resistance. Diabetologia 2023; 66(3):461-471.

78. Albalak G, Stijntjes M, Wijsman CA, et al. Timing of objectively-collected physical activity in relation to body weight and metabolic health in sedentary older people: a cross-sectional and prospective analysis. Int J Obes (Lond) 2022; 46(3):515-522.

79. Eastman CI, Hoese EK, Youngstedt SD, Liu L. Phase-shifting human circadian rhythms with exercise during the night shift. Physiol Behav 1995; 58(6):1287-1291.

80. Savikj M, Gabriel BM, Alm PS, et al. Afternoon exercise is more efficacious than morning exercise at improving blood glucose levels in individuals with type 2 diabetes: a randomised crossover trial. Diabetologia 2019; 62(2):233-237.

81. Ruegemer, J., Squieres, Ray W., Marsh, Harold M., Haymond, Morey W., Cryer, Philip E., Rizza, Robert A., Miles JM. Differences Between Prebreakfast and Late Afternoon Lgycemic Responses to Exercise in IDDM Patients. J Chem Inf Model 2019; 53(9):1689-1699.

82. Toghi-Eshghi SR, Yardley JE. Morning (Fasting) vs Afternoon Resistance Exercise in Individuals with Type 1 Diabetes: A Randomized Crossover Study. Journal of Clinical Endocrinology and Metabolism 2019; 104(11):5217-5224.

83. Moholdt T, Parr EB, Devlin BL, Debik J, Giskeødegård G, Hawley JA. The effect of morning vs evening exercise training on glycaemic control and serum metabolites in overweight/obese men: a randomised trial. Diabetologia 2021; 64(9):2061-2076.

84. Morales-Palomo F, Moreno-Cabañas A, Alvarez-Jimenez L, Mora-Gonzalez D, Ortega JF, Mora-Rodriguez R. Efficacy of morning versus afternoon aerobic exercise training on reducing metabolic syndrome components: A randomized controlled trial Key points. J Physiol 2023:1-15.

85. Alizadeh Z, Younespour S, Rajabian Tabesh M, Haghravan S. Comparison between the effect of 6 weeks of morning or evening aerobic exercise on appetite and anthropometric indices: a randomized controlled trial. Clin Obes 2017; 7(3):157-165.

86. Arciero PJ, Ives SJ, Mohr AE, et al. Morning Exercise Reduces Abdominal Fat and Blood Pressure in Women; Evening Exercise Increases Muscular Performance in Women and Lowers Blood Pressure in Men. Front Physiol 2022; 13:893783.

87. Handelsman DJ, Hirschberg AL, Bermon S. Circulating Testosterone as the Hormonal Basis of Sex Differences in Athletic Performance. Endocr Rev 2018; 39(5):803.

88. Hunter SK, Angadi SS, Bhargava A, et al. The Biological Basis of Sex Differences in Athletic Performance: Consensus Statement for the American College of Sports Medicine. Transl J Am Coll Sports Med 2023; 8(4):1-33.

89. Oosthuyse T, Bosch AN. Oestrogen's regulation of fat metabolism during exercise and gender specific effects. Curr Opin Pharmacol 2012; 12(3):363-371.

90. McNulty KL, Elliott-Sale KJ, Dolan E, et al. The Effects of Menstrual Cycle Phase on Exercise Performance in Eumenorrheic Women: A Systematic Review and Meta-Analysis. Sports Med 2020; 50(10):1813.

91. Cowan SM, Kemp JL, Ardern CL, et al. Sport and exercise medicine/physiotherapy publishing has a gender/sex equity problem: we need action now! Br J Sports Med 2023; 57(7):401-407.

92. Meignié A, Toussaint JF, Antero J, Fr JA. Dealing with menstrual cycle in sport: stop finding excuses to exclude women from research. Eur J Appl Physiol 2022; 122(3):651-661.

93. James JJ, Klevenow EA, Atkinson MA, et al. Underrepresentation of women in exercise science and physiology research is associated with authorship gender. J Appl Physiol 2023; 135(4):932-942.

AIMS

The overall aim of this International Doctoral Thesis is to investigate the diurnal variation of the effect of exercise on glucose regulation and energy metabolism in men and women.

- **Specific aim 1.** To analyze the acute impact of time of the day of exercise on cardiovascular disease risk factors in men and women (Chapter 3).
- **Specific aim 2.** To determine the impact of an acute moderate-intensity aerobic exercise session performed in the morning vs. evening on glucose regulation and energy metabolism in healthy men and women (Chapter 4).
- **Specific aim 3.** To describe the effect of an acute moderate-intensity aerobic exercise session performed in the morning vs. evening on mitochondrial function, mitochondrial proteome, and transcriptome in the skeletal muscle in healthy men and women (Chapter 4).
- **Specific aim 4.** To determine the influence of an acute moderate-intensity aerobic exercise session performed in the morning vs. evening on perceived appetite feelings, Lac-Phe, CNDP2, lactate, and IL-6 in healthy men and women (Chapter 5).

HYPOTHESES

The main hypothesis of this International Doctoral thesis is that time of exercise plays an important role glucose regulation and energy metabolism in humans. Specifically, we hypothesize that:

- The time-of-day influences the response to exercise of cardiovascular disease risk factors (e.g., blood pressure, blood glucose, and blood lipids).
- Exercise has major impact on glucose regulation and energy metabolism when performed in the evening in healthy humans. This diurnal variation may differ between men and women.
- The time-of-day of exercise influences the adaptations relative to mitochondrial function, mitochondrial proteome, and transcriptome in the skeletal muscle in healthy humans. This diurnal variation may differ between men and women.
- The time-of-day influences the response to exercise of energy regulatory metabolites (i.e., Lac-Phe, CNDP2, lactate, and IL-6) and consequently, the response to exercise of perceived appetite feelings. This diurnal variation may differ between men and women.

MATERIALS AND METHODS

Chapter 2. Methodological overview of the DIVA study

The Diurnal Variation of Exercise on Metabolic Health (DIVA) study is a randomised crossover study (ClinicalTrials.gov ID: NCT05369715) designed to investigate the the impact of an acute moderate-intensity aerobic exercise session performed in the morning vs. evening on glucose regulation and energy metabolism in healthy men and women, and to determine the underlying molecular mechanisms in the skeletal muscle.

After preliminary examination (i.e, medical screening, sociodemographic, lifestyle and body composition data) and a maximal effort test, 18 men and 17 women performed a 60 min steady-state moderate (i.e., 65% HR reserve) exercise bout in the morning (i.e., 11:30 a.m.) and evening (i.e., 6:30 p.m.) in a randomised and counterbalanced order. Visits were separated by at least 4 days and the same procedures were followed on both visits. Participants were wearing a continuous glucose monitor until the end of the study to evaluate interstitial glucose continually from the previous day to morning and evening exercise to the two subsequent days. During morning and evening exercise trials, gas exchange was measured at rest before exercise (i.e., pre), during exercise and after exercise (i.e., post) to determine substrate oxidation and energy expenditure. Serum and plasma samples and perceived appetite feelings were evaluated pre-exercise, post-exercise and 90 min post-exercise. In a subsample of 6 men and 8 women, biopsies from the vastus lateralis' distal part of the quadriceps were collected pre-exercise and post-exercise. Skeletal muscle samples were used to study fresh mitochondrial oxygen consumption, mitochondrial protein supercomplexes, whole fiber transcriptomics, and mitochondrial proteomics.

For women the phase of their menstrual cycle was registered. On the previous day to morning and evening trials, physical activity was controlled, and diet and fasting hours were standardized. On the visit day, 4 hours before exercise participants consumed a standardized meal. The subsequent meal was again standardized and consumed within 1 hour after leaving the laboratory. Participants were instructed to control diet and physical activity the two subsequent days to the first exercise condition, either morning or evening, and replicate them the two subsequent days to the second exercise condition.

The trial strictly followed CONSORT guidelines (http://www.consort-statement.org/consort-statement). Study design, protocols, and informed consent procedure were performed according to the Declaration of Helsinki, and it was approved by the Human Research Ethics Committee of the University of Granada and the Provincial

Human Research Ethics Committee (Junta de Andalucía, ref. 1288-N-20). All participants gave their oral and written informed consent. The evaluations were placed between February and July 2022, in the same setting [Instituto Mixto Deporte y Salud (iMUDS) at the University of Granada and Hospital Universitario San Cecilio, Granada, Spain] and were performed by the same investigators.

RESULTS AND DISCUSSION

Chapter 3. Time of the day of exercise impact on cardiovascular disease risk factors in adults: a systematic review and meta-analysis

1. Abstract

Objective: To compare the effect of a single bout of morning vs. evening exercise on cardiovascular risk factors in adults. Design: Systematic review and meta-analysis. Methods: A systematic search of studies was conducted using PubMed and Web of Science from inception to June 2022. Selected studies accomplished the following criteria: crossover design, acute effect of exercise, blood pressure, blood glucose, and/or blood lipids as the study's endpoint, a washout period of at least 24 hours, and adults. Meta-analysis was performed by analysing: 1) separated effect of morning and evening exercise (pre vs. post); and 2) comparison between morning and evening exercise. Results: A total of 11 studies were included for systolic and diastolic blood pressure and 10 studies for blood glucose. Meta-analysis revealed no significant difference between morning vs. evening exercise for systolic blood pressure (g Δ= 0.02), diastolic blood pressure (g Δ= 0.01), or blood glucose (g Δ= 0.15). Analysis of moderator variables (age, BMI, sex, health status, intensity and duration of exercise, and hour within the morning or evening) showed no significant morning vs. evening effect. Conclusion: Overall, we found no influence of the time of the day on the acute effect of exercise on blood pressure neither on blood glucose.

2. Introduction

Mammalian cells possess an internal molecular clock that controls metabolic processes through the so-called “clock genes”, regulated in a transcriptional-translational feedback loop[1]. This feedback loop consists of an autonomous central clock placed in the suprachiasmatic nucleus of the hypothalamus that, affected by endogenous and external cues (e.g., exercise), regulates peripheral clocks[2]. In animal models, the alteration of the molecular clock has been associated with the occurrence of obesity and type II diabetes mellitus[3]. Similarly, in humans, shifted sleep patterns seem to interfere with several metabolic pathways[4]. Shifted working, short sleep duration, exposure to artificial light, inadequate eating time window, and lack of physical activity, are some characteristics of the modern lifestyle that contributes to the occurrence and worsening of cardiovascular disease (CVD)[5].

Exercise is a well-known protective factor against CVD and can reduce all-cause mortality by 50%[6]. Recent epidemiological studies highlight the relevance of the time of exercise on

its cardiovascular and metabolic effects[7–9]. To understand these adaptations, it is of interest to define the physiological acute response to exercise at different times of the day. Savikj et al.[10] reported that a single bout of evening high-intensity exercise was more efficacious at improving blood glucose in men with type 2 diabetes than morning exercise (although they did not control previous diet). Jones et al.[11,12] found that the acute hypotensive effect of exercise was more significant in the evening compared to morning in normotensive men. In contrast, Brito et al.[13] found a greater hypotensive effect of morning than evening exercise in pre-hypertensive men. Given the current contradiction would be of great interest to systematically review the literature and synthesize the results with a standardized protocol (i.e., meta-analysis) to respond whether exercise has a different impact on CVD risk factors when performed during the morning or the evening. This finding would be of clinical and public health interest contributing to optimize the effects of exercise in the prevention of CVD. Thus, the objective of this systematic review and meta-analysis is to analyse the time of the day of exercise-induced effects on CVD risk factors in adults.

3. Materials and methods

To follow the quality of the design, implementation, and reporting of this meta-analysis, we adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[14] (see Table S1) and relevant methodological references[15–17] throughout the entire process. This systematic review and meta-analysis protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO ID: CRD42021283350).

3.1. Search strategy and information sources

We performed a systematic search of studies in PubMed and Web of Science databases, from inception to June 2022. We used the following terms for that purpose: exercise, time of day, diurnal variation, circadian rhythm, morning, afternoon, evening, glucose, triglycerides, blood lipids, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, total cholesterol, blood pressure with synonymous and truncation operators adapted to each database (see Table 1 for specific search strategies).

3.2. Eligibility criteria

Studies were eligible to be included upon meeting the following criteria: (a) crossover design studies investigating the effect of morning vs. evening exercise; (b) to include at

least one cardiovascular risk factor as a study's endpoint (i.e., blood glucose, blood pressure, total cholesterol, HDL, LDL, or triglycerides); (c) to investigate the acute effect of exercise before and in between 15 to 60 minutes; (d) to consider a washout period of at least 24 hours between exercise sessions; (e) to include adults above 18 years old regardless of their health or physical condition; (f) manuscripts written in English or Spanish; (h) to provide statistical indicators that allow calculating the effect size; and (j) not to include any type of drug, dietary supplement or equipment before exercising that could affect exercise effects.

Table 1. Search strategy used in each database.

Database	Search Strategy
Pubmed	(exercise [Title / Abstract]) AND ("different times of day" [Title / Abstract] OR "time-of-day" [Title / Abstract] OR "time of day" [Title / Abstract] OR "diurnal variation "[Title / Abstract] OR" circadian rhythm "[Title / Abstract] OR (morning [Title / Abstract] AND (afternoon [Title / Abstract] OR evening [Title / Abstract]))) AND (" glucose "[Title / Abstract] OR "CGM" [Title / Abstract] OR "glycaemia" [Title / Abstract] OR "triglycerides" [Title / Abstract] OR "blood lipids" [Title / Abstract] OR "HDL" [Title / Abstract] OR "total cholesterol" [Title / Abstract] OR "LDL" [Title / Abstract] OR "blood pressure" [Title / Abstract] OR "diastolic" [Title / Abstract] OR "systolic" [Title / Abstract]) Filters: Journal Article, Humans, English, Spanish
Web of Science	(TS = (exercise) AND (TS = ("different times of day" OR "time-of-day" OR "time of day" OR "diurnal variation" OR "circadian rhythm" OR (morning AND (afternoon OR evening))))) AND TS = ("glucose" OR "CGM" OR "glycaemia" OR "triglycerides" OR "blood lipids" OR "HDL *" OR "total cholesterol" OR "LDL*" OR "blood pressure" OR "diastolic" OR "systolic") Refined by: LANGUAGES: (ENGLISH OR SPANISH) AND TYPES OF DOCUMENTS: (ARTICLE)

3.3. Selection and data collection process

Based on the selection criteria, screening by title and abstract was independently performed by two authors (R.S.L. and F.A.G.) using EndNote. Disagreements between

authors were resolved by discussion and, if needed, a third author's (J.R.R.) final decision was required. Full manuscripts of potential studies were obtained and screened for final inclusion and data extraction following the same procedure. Data extraction of the included studies was performed through a codebook and a coding protocol previously created for this purpose in a standardized form. The standardized form included the authors' names, and year of publication (extrinsic variables); participants and treatment characteristics (substantive variables); and methodological variables, in addition to the study outcomes. We contacted the authors of studies when required data of their works were not explicitly found. Outcomes of interest were systolic (SBP), diastolic (DBP) and mean blood pressure (MBP), blood glucose, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides. We extracted data from pre and 15 to 60 minutes post-exercise at both moments of the day (morning and evening). If a study had several points of measurements post-exercise, data closer to 60 minutes post were selected. We discarded data coming from immediately post-exercise measurements because exercise is a stressor that acutely elevates blood glucose and blood pressure while occurring[18,19], and could mask the cardiovascular benefits coming later. Exercise performed from wake up to 12:00 was considered “morning exercise”, and the one performed from 14:00 to bedtime was considered “evening exercise”. If a study was designed with several morning and/or evening trials (e.g., 8:00 vs. 12:00 vs. 15:00 vs. 18:00), we chose for morning the one closer to 8:00 and for evening, the one closer to 18:00. These were the most common exercise times in the included studies and we decided to harmonize the data as much as possible. Lastly, if the same data/study was used in different original articles for different purposes, only the report that provides more detailed information on the subject of this systematic review-meta-analysis was included.

3.4. Study risk of bias assessment

Included studies were assessed for crossover design methodological quality using relevant items, based on the Cochrane handbook and expert comments[20]. Nine standard items were used to evaluate the risk of bias: 1) to follow an appropriate cross-over design; 2) to randomize the order of receiving treatment; 3) to account for the carry-over effects; 4) participants blinding (this was always considered as low risk of bias since morning and evening conditions cannot be blinded); 5) to apply blinding methods to researchers; 6) if an appropriate statistical analysis was performed; 7) to provide information about incomplete outcome data; 8) to avoid selective outcome reporting; and 9) to measure outcomes appropriately (i.e., to control previous conditions to exercise: physical activity,

fasting hours and diet). All of them were judged as high, unclear, or low risk of bias based on the study methods reported in the original articles.

3.5. Effect measures and synthesis methods

We calculated two effect sizes: 1) separated effect of morning and evening exercise (pre vs. post); and 2) comparison between morning and evening exercise. To compare the separated effect of exercise during both morning and evening, we followed the approach by Kebede et al.[21]. First, we calculated Cohen's d for repeated measures (Cohen's d_{RM}) taking into consideration baseline imbalance and pre/post correlation[22]. Pre/post correlations were computed from standard deviations (SD_{pre} and SD_{post}), and standard deviation values for change scores[21]. Second, we calculated the Hedges' g for repeated measures since all of the studies included less than 20 participants[22]. To compare morning and evening's effect sizes we used the formula reported by Madeyski et al.[23]. Standard errors of the effect sizes presented in the forest plot were calculated from the variance following previously published formulas[24]. Table 2 summarizes the equations used to calculate the effect sizes. The effect sizes can be interpreted according to the standard benchmarks: values around 0.2 are considered a small effect size, 0.5 is considered a medium effect, and above 0.8 is considered a large effect size[25]. Using R software, the random-effect model of the inverse variance method was used to calculate the pooled Hedges' g and the corresponding 95% CI. Heterogeneity was assessed with the Higgins I^2 statistic and P values, being classified as not important (0%–40%), moderate (30%–50%), substantial (50%–75%), or considerable (75%–100%) [26].

We also performed a sensitivity analysis to determine whether potential moderators are influencing the effect sizes. For that purpose, we used meta-regression analyses for continuous variables and analyses of variance for the categorical variables. The moderators included were sex, BMI, age, health status (i.e., healthy and unhealthy), hour in the morning, hour in the evening, duration of exercise, and intensity (i.e., low to moderate and moderate to high) of exercise. Moderation of the hour within the morning and the hour within the evening were only possible to be analysed from the separate meta-analyses of morning and evening exercise. Potential publication bias was assessed using Egger's test and the Rosenthal method (fail-safe N index).

Table 2. Equations used for the calculation of effect sizes.

	Effect size of morning and afternoon pre vs. post	Effect size of morning vs. afternoon
Standardized effect size (Hedges' g)	$g_{RM} = Cohen's\ d_{RM} \times \left(1 - \frac{3}{4(n+n)-9}\right)$ $Cohen's\ d_{RM} = \frac{M_{diff}}{S_{diff}} \times \sqrt{2(1-r)}$	$g_{RM} = \frac{c(df) \times \left(g_{RM\ morning} - g_{RM\ afternoon}\right)}{SD_{pooled}}$ $c(df) \approx 1 - \frac{3}{4df-1}$
Pooled standard deviation	$S_{diff} = \sqrt{SD_{pre}^2 + SD_{post}^2 - 2 \times r \times SD_{pre} \times SD_{post}}$	$SD_{pooled} = \sqrt{\frac{\left(SD_{morning}^2 + SD_{afternoon}^2\right)}{2}}$
R coefficient (r)	$r = \frac{SD_{pre}^2 \times SD_{post}^2 - \left(SD_{pre} - SD_{pre}\right)^2}{2 \times SD_{pre}^2 \times SD_{post}^2}$	Not required
Variance	$\sigma^2 = \frac{df+1}{df-1} \times \frac{2}{n}\left(1 + \frac{{g_{RM}}^2}{8}\right)$	$\sigma^2 = \frac{df+1}{df-1} \times \frac{2}{n}\left(1 + \frac{{g_{RM}}^2}{8}\right)$

4. Results

4.1. Search results

The systematic search yielded 1273 studies. After removal of duplicates and screening by title and abstract, 177 eligible full-text documents were evaluated for inclusion in the meta-analysis. The flowchart of the search and selection of studies is shown in Figure 5. A total of 28 crossover studies[10,11,33–42,12,43–50,13,27–32] were selected for inclusion. Of those, 6 were excluded because they did not provide statistical data to calculate effect size or full text was not available. Thus, a total of 22 studies were finally included in the meta-analysis.

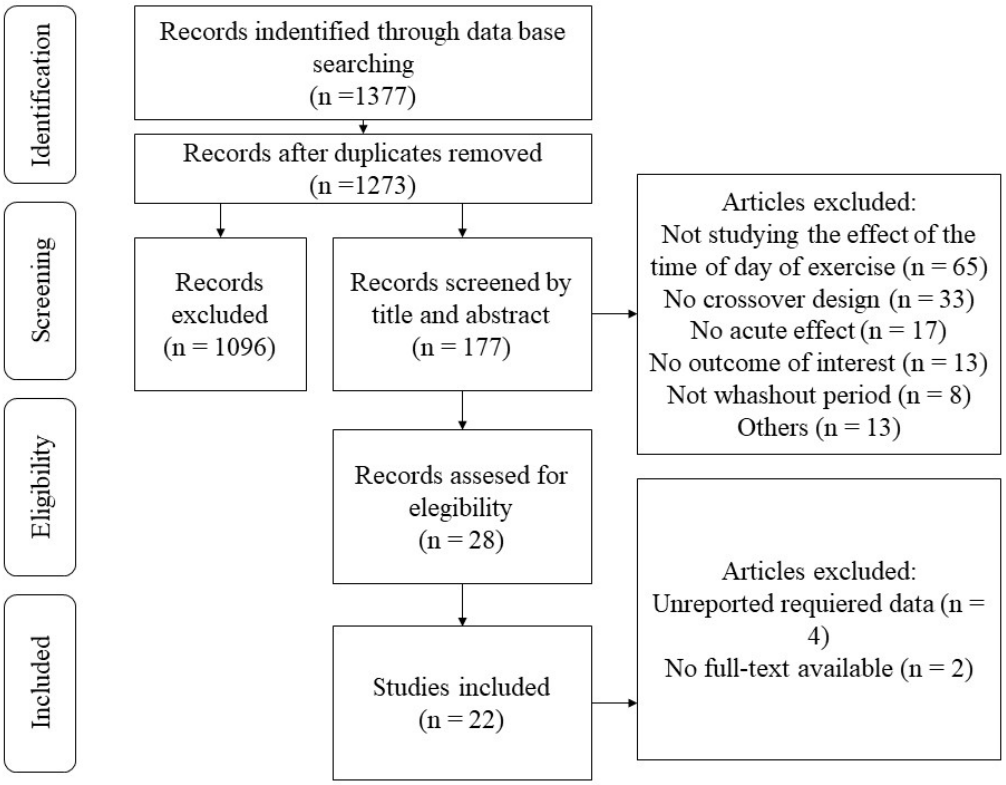


Figure 5. Flowchart of the search and selection of studies

4.2. Studies characteristics

The general characteristics of all included studies are summarized in Table 3. For SBP and DBP, the total sample was composed of 144 participants (9.03% women), the median age was of 29 years (Q_1 = 22.3, Q_2 = 29, Q_3 = 49), and the mean body mass index (BMI) was 25.95 kg/m^2 ± 3.62. Most of the study's participants were healthy (61.11%), being the rest hypertensives (27.78%) or pre-hypertensives (11.11%). For MBP, the total sample was composed of 103 participants, 0% women, with a median age of 29 years (Q_1 = 26, Q_2 = 29, Q_3 = 49) and a BMI of 26.38 ± 3.32 kg/m^2. Most of the participants were healthy (56.31%), the others were hypertensives (28.16%) or pre-hypertensives (15.53%). Regarding blood glucose, the total sample was composed of 91 participants, 27.47% women, with a median age of 30.5 years (Q_1 = 25, Q_2 = 30.5, Q_3 = 49) and a mean BMI of 25.61 ± 1.93 kg/m^2. Part of the participants was healthy (42.86%) or had no metabolic disease, i.e., mild sleep apnea (13.19%)[44], the others presented type 1 or type 2 diabetes (43.96%). With regards to blood lipids, we did not find enough studies to meta-analyse this outcome: only 1 of the studies met the inclusion criteria determining blood triglycerides[33].

Considering the total of the included studies, most of them were originally from the United Kingdom (n = 6), the United States of America (n = 4), and Brazil (n = 3); the others were conducted in Australia, Canada, Italy, Japan, Sweden, Tunisia, and Turkey. Exercise interventions were mainly aerobic exercise sessions (n = 19)[10,11,40,42–44,46–48,51,52,12,13,33,35–39]; the rest applied strength training (n = 2)[41,49] or combined strength and aerobic exercise (n = 1)[34]; exercise sessions had a mean duration of 27.25 ± 15.03 minutes. Respectively, median morning and evening hours for SBP and DBP were 8:00 a.m. (min = 7:00, max = 9:00 a.m.) and 6:00 p.m. (min = 4:00, max = 8:00 p.m.). For blood glucose, median hours were 7:00 a.m. (min = 6:00, max = 8:00 a.m.) and 4:00 p.m. (min = 3:00, max 7:00 p.m.). Outcomes were evaluated at rest previous to exercise and post-exercise within a period of 15 to 60 minutes.

Table 3. Overview of acute effect of time of day of exercise studies on CVD risk factors

Acute effect of time of day of exercise on systolic, diastolic and mean blood pressure

Study	N (sex:)	Age (SD)	BMI (SD)	Chrono-type	Health and training status	Medication	Design	Exercise intervention	Previous conditions	Time of the day	Main outcome	Minutes post exercise	Main findings
Aldemir and Kilic, 2005	10 (male)	27 (1.6)	23.9 (0.7)	Not reported	Healthy, moderately active	No	Randomized crossover	Submaximal exercise test: 1 bout x 30 min on cycle ergometer (intensity: 70%VO2max)	Diet, fasting, or previous activity not reported	07:30 vs 17:30	Mean blood Pressure Blood triglycerides	30	Although blood pressure decreased in response to submaximal exercise, there was no time of day differences neither at baseline, post-exercise nor recovery. Blood triglycerides decreased after evening exercise but not after morning exercise.
Azevedo et al, 2017	11 (female)	57 (5.1)	30.7 (3.9)	Not reported	Hyper-tension, training status not reported	anti-hypertensive treatment	Randomized crossover with control condition	Strength training: 8 maximum repetitions test: (1 × 10 repetitions at 50% load) + Moderate-to-severe cycling for 20 min at 7–8 SPE scale	Diet, fasting or previous activity not reported. Caffeine intake was not suspended	8:00 vs 18:00	Systolic and diastolic blood pressure	60	No differences were observed for post-exercise hypotension between morning and evening exercise. Diastolic blood pressure did not show reductions after exercise.
Boukelia et al, 2018	12 (male)	33 (5)	21.9 (1.2)	Not reported	Healthy, athletes	No	Randomized crossover	10 km treadmill time trial (controlled environmental conditions: 28°C, 70% relative humidity) Intensity not reported	Diet, fasting, or previous activity not reported	9:00 vs 18:00	Systolic and diastolic blood pressure	60	No differences were found in mean blood pressure reduction after exercise between the morning and the evening trial.
Bousseta et al, 2017	11 (male)	21.82 (0.5)	23.4 (1.3)	Not reported	Healthy, trained	No	Randomized crossover	Yo–Yo tests level 1: 20-m shuttle runs at increasing velocities until exhaustion.	Not controlling previous diet, standardized fasting conditions (at least 6 h). Inactivity the day before.	8:00 vs 18:00	Systolic and diastolic blood pressure	60	No differences were found in systolic or diastolic blood pressure reduction after exercise between the morning and the evening trial. Systolic blood pressure was higher in the polluted area at both times of day.
Brito et al, 2015	16 (male)	32 .0 (7.0)	28.9 (2.8)	Interme-diate types	Pre-hyper-tension. Sedentary to moderately active.	No	Randomized crossover with control condition	1 bout x 45 min on cycle ergometer (intensity: 50% VO2peak)	Standardized diet and fasting (0.5h). Inactivity the day before.	09:00 vs 18:30	Systolic, diastolic and mean blood pressure	45	Morning aerobic exercise had an important and greater hypotensive effect than evening exercise

Study	N (sex:)	Age (SD)	BMI (SD)	Chrono-type	Health and training status	Medication	Design	Exercise intervention	Previous conditions	Time of the day	Main outcome	Minutes post exercise	Main findings
Brito et al, 2020	14 (male) for ACEi, 15 (male) for ARB	50 (8) for ACEi, 49 (8) for ARB	30.9 (3.6) for ACEi, 29.8 (4.1) for ARB	Interme-diate types	Hyper-tension. Sedentary	Group 1: angiotensin-converting enzyme inhibitors (ACEi) Group 2: angiotensin II receptor blockers (ARB)	Randomized crossover	Maximal cardiopulmonary exercise test on cycle ergometer (increasing workload 15W every minute until exhaustion).	Abstain from caffeine and alcohol the day before, standardized previous fasting (2h). Inactivity the day before.	8:00 vs 21:00	Systolic, diastolic and mean blood pressure	30	Exercise produced bigger hypotensive effect after evening exercise compared to morning in hypertensives receiving ARB, but not ACEi. The anti-hypertensive drug can influence the diurnal variation of post-exercise hypotension.
Chan-Dewar et al, 2012	10 (male n= 10, female n= 2)	24 (3)	23.5 (1.2)	Not reported	Healthy, trained	No	Randomized crossover	40 km cycling on a computerised cycle training system (intensity: 90-100% lactate threshold, 74-90% HRmax)	Standardized previous diet and fasting (1.5 h). Inactivity the day before.	8:00 vs 18:00	Systolic and diastolic blood pressure	60	No differences were found in systolic or diastolic blood pressure reduction after a high-intense exercise between the morning and the evening trial.
Di Blasio et al, 2010	28 (male)	25 (2)	24.2 (1.72)	Not reported	Healthy, sedentary	No	Randomized crossover	1 bout of cycling on cycle ergometer (intensity: 10 min. at 55% HRmax, 35 min. at 70% HRmax, 5 min. cool down)	Abstain from caffein and alcohol the day before, standardized fasting conditions (3 h). No info. about activity the day before.	9:00 vs 14:00 vs 18:30	Mean blood pressure	30	During exercise in the early evening there was a trend to decrease for diastolic blood pressure compared to morning. But there was no difference for systolic blood pressure.
Focht et al, 2009	21 (male)	21.4 (2.5)	Not reported	Mixed	Healthy, recrea-tionally active	Not reported	Randomized crossover	45 min of strength training: 4 exercises completed for 3 sets of 10 repetitions at 75% 1 RM	Abstain from caffeine and alcohol the day before. Not report info. about fasting. Inactivity the day before.	7:00 vs 19:00	Systolic and diastolic blood pressure	15	Only diastolic blood pressure decreased 15 minutes after exercise, but there were no differences in the response relative to the time of day.
Jones et al, 2008	12 (male)	26.0 (5.0)	23.5 (0.7)	Not reported	Healthy, recrea-tionally active	No	Randomized crossover	30 min cycling on cycle ergometer (intensity: 70% VO2peak)	Abstain caffein and alcohol the day before, standardized previous fasting conditions (4 h). Inactivity the day before.	08:00 vs 16:00	Systolic, diastolic and mean blood pressure	20	Afternoon aerobic exercise produced a bigger hypotensive effect than morning exercise.

Study	N (sex:)	Age (SD)	BMI (SD)	Chrono-type	Health and training status	Medication	Design	Exercise intervention	Previous conditions	Time of the day	Main outcome	Minutes post exercise	Main findings
Jones et al, 2009	8 (male)	29.0 (7.0)	26.6 (9.5)	Not reported	Healthy, recrea-tionally active	No	Randomized crossover	Continuous steady-state: 30 min cycling on cycle ergometer (intensity: 70%VO2peak) Intermittent steady-state: 3 bouts of 10 min cycling on cycle ergometer separated by 10 min resting periods (intensity: 70% VO2peak)	Abstain from caffein and alcohol the day before, standardized previous fasting conditions (4 h). Inactivity the day before.	08:00 vs 16:00	Systolic, diastolic and mean blood pressure	20	Afternoon aerobic exercise decreased greater mean and systolic blood pressure compared to morning exercise. This diurnal variation was less marked following intermittent than continuous exercise.
O'Connor et al, 1992	12 (male)	22.3 (2.7)	23.3 (0.1)	Mixed but not definitivel y morning or evening types	Not reported	Not reported	Randomized crossover	Submaximal exercise test: 20 min running on treadmill (intensity: 70% VO2max)	Not reported	8:00 vs 16:00 vs 20:00	Systolic and diastolic blood pressure	20	Post-exercise hypotension was independent of the time of the day that exercise was performed.

Table 3 (continue). Overview of acute effect of time of day of exercise studies on CVD risk factors

Acute effect of time of day of exercise on blood glucose

Study	N (sex)	Age (SD)	BMI (SD)	Chrono-type	Health and training status	Medication	Design	Exercise intervention	Previous conditions	Time of the day	Main outcome	Minutes postexercise	Main findings
Fernandes et al, 2014	9 (male)	31 (7.3)	23.8 (1.7)	Intermediate (n =5) or moderate morning (n =4)	Healthy, trained	Not reported	Randomized crossover	1000-m cycling time trial in the shortest time possible	Standardized diet and previous fasting (6 h). Inactivity the day before.	08:00 vs 18:00	Blood glucose	60	Blood glucose showed a tendency to increase greater after morning exercise compared to evening, accompanied with a more exacerbated response to exercise of norepinephrine at the same time of the day.
Galliven et al, 1997	7 (female)	29 (2.6)	23.7 (4.5)	Not reported	Healthy, mixed training status	No	Randomized crossover	20 min running on treadmill (intensity: 5 min at 50% VO2max, 10 min at 70% VO2max, 5 min at 90% VO2max)	Only abstain from caffeine and alcohol the day before, standardized fasting and fasting (6 h). Inactivity the day before	07:00 vs 15:00	Blood glucose	60	There were no differences for the blood glucose response to exercise between morning and evening trials.
Hobson et al, 2009	7 (male)	24.0 (2.0)	24.2 (3.1)	Not reported	Healthy, recreationally active	Not reported	Randomized crossover	1 bout of cycling on a cycle ergometer until exhaustion (intensity: 65% VO2max) Environmental conditions: 35.1 (0.4) ºC and 60 (4) % relative humidity.	Standardized previous diet and fasting (6 h). Inactivity the day before	06:45 vs 18:45	Blood glucose	15	Blood glucose levels were not reduced after exercise, and there were no differences between morning and evening trials.
Larsen et al, 2019	11 (male)	49 (5)	28 (3)	Not reported	Mild sleep apnoea, sedentary	No	Randomized crossover	30 min HIIT (intensity: 60 work s at 100% VO2max, 240 s rest at 50% VO2peak)	Controlled previous diet, not standardized fasting (overnight for morning, 3 h for evening).	6:00 vs 15:00 vs 19:00	Blood glucose	30	After morning exercise blood glucose showed bigger increase than after evening trials.
McIver et al, 2019	12 (male)	25.0 (3.0)	26.0 (4.0)	Intermediate	Healthy, recreationally active	No	Randomized crossover	45 min walking on treadmill (intensity: 55% VO2peak)	Abstain from alcohol and caffein the day before, standardized previous fasting (8 h). Inactivity the day before.	08:00 vs 15:00	Blood glucose	30	There were no diurnal differences for blood glucose in response to exercise.

Study	N (sex)	Age (SD)	BMI (SD)	Chrono-type	Health and training status	Medication	Design	Exercise intervention	Previous conditions	Time of the day	Main outcome	Minutes postexercise	Main findings
Munan et al, 2020	14 (male n= 8, female n= 6)	65 (9)	27.2 (3.5)	Not reported	Type 2 diabetes, sedentary	Not insulin or corticosteroids. Metformin (n= 12).	Randomized crossover	40 min walking on treadmill (intensity: 5 km/h with 0.5% grade)	Standardized previous diet but not fasting (overnight for morning, 3 h for afternoon, 20 min for evening). Inactivity the day before.	Not reported	Blood glucose	30	The afternoon exercise condition had the largest decrease in blood glucose after exercise. No significant diurnal differences were found between trials in mean 24 h continuously monitored glucose.
Ruegemer et al, 1990	6 (male n= 3, female n= 3)	30 (9.8)	Not reporter	Not reported	Type 1 diabetes, recreationally active	Ultralente-based intensive insulin therapy	Randomized crossover with control condition	30 min cycling on cycle ergometer (intensity: 60% VO2max)	Standardized diet but not fasting hours (9 h for morning, 4 h for evening). No information about activity the day before.	7:00 vs 16:00	Blood glucose	60	Exercise in the morning produced a significant increase in blood glucose, while this hyperglycaemic effect was absent after evening exercise.
Savikj et al, 2018	11 (male)	60 (2)	27.5 (0.6)	Not reported	Type 2 diabetes, sedentary	Dietary treatment or metformin	Randomized crossover	HIIT: 6 bouts x 1 min work (intensity > 220 W) + 1 min rest (minimal load).	Not controlled previous diet, not standardized fasting (1 h for morning, 3 h for evening)	08:00 vs 16:00	Blood glucose, HDL, triglycerides	60	Afternoon HIIT reduced blood glucose. Morning HIIT induced an increase of blood glucose. There was no time of day effect neither in HDL nor in triglycerides, measured before and after 2 weeks of exercise.
Tanaka et al, 2021	11 (male)	24.5 (2.8)	22.3 (1.1)	Not reported	Recreationally active	No	Randomized repeated measures	60 min cycling on cycle ergometer (intensity: 60% VO2max)	Standarized diet but not fasting hours (overnight fast for morning, 3 h for evening)	7:00 vs 16:00	Blood glucose	60	The effect of morning versus afternoon exercise was not significantly different during the 60 minutes period after the test. However, blood glucose increased greater after a given post-exercise meal in the morning compared to afternoon.
Toghi-Eshghi et al, 2019	12 (male n= 3, female n= 9)	31 6 (8.9)	26.6 (3.8)	Not reported	Type 1 diabetes, recreationally active	Insulin pump (n = 8), insulin injections (n= 4)	Randomized crossover	43 min of strength training: 3 sets involving major muscle groups x 8 repetitions (intensity: 8 RM)	Controlled previous diet, not standardized fasting (overnight for morning, 3 h for evening). Inactivity the day before.	07:00 vs 17:00	Blood glucose	60	Morning exercise led to an increase in blood glucose meanwhile afternoon exercise lead to a decrease.

4.3. Acute effect of morning vs. evening exercise on cardiovascular disease risk factors

Meta-analysis of the acute effect of the time of day of exercise on SPB (11 studies), DBP (11 studies), and blood glucose (10 studies) are shown in Figure 6. Meta-analysis of the acute effect of the time of day of exercise on MPB (6 studies) is shown in Figure S3. We found no significant differences in the morning vs. evening effect of exercise on SBP (Hedges' g Δ: 0.02 favour morning [-0.22, 0.26]; p = 0.88), DBP (Hedges' g Δ: 0.01 favour morning [-0.26, 0.23]; p = 0.95) or blood glucose (Hedges' g Δ: 0.15 [-0.22, 0.53]; p = 0.42). Mean changes expressed in mmHg and mmol/L are reported in Table 4. Heterogeneity was low for SBP and DBP ($I^2 \leq 21.42\%$, $p > 0.05$), and moderate for blood glucose ($I^2 = 34.73\%$, p= 0.13).

Blood lipids could not be meta-analysed because only one study was reporting this outcome. Aldemir et al.[33] explored the diurnal variation of the effect of exercise on blood lipids, specifically on triglycerides. They reported that triglycerides levels 30 minutes after evening exercise were significantly lower from baseline (from 20.07 ± 5.08 to 15.52 ± 4.41 UI/l) but not after morning exercise (from 22.61 ± 7.49 to 20.20 ± 6.29 UI/l).

4.4. Analysis of potential moderator variables

The moderation from sex, BMI, age, health status, exercise intensity, and duration did not reach statistical significance for any outcome when comparing morning vs. evening effects (all $P > 0.05$).

From the separate meta-analyses of morning and evening exercise we found that none of the outcomes were affected by the hour within the morning or within the evening in which exercise was performed ($P \geq 0.05$). For blood glucose in the morning, we observed that the exercise-induced increase was not significant in healthy participants ($g = 0.27$) but significant in non-healthy participants (i.e., type 1 and type 2 diabetes, and mild sleep apnoea) ($g = 0.74$).

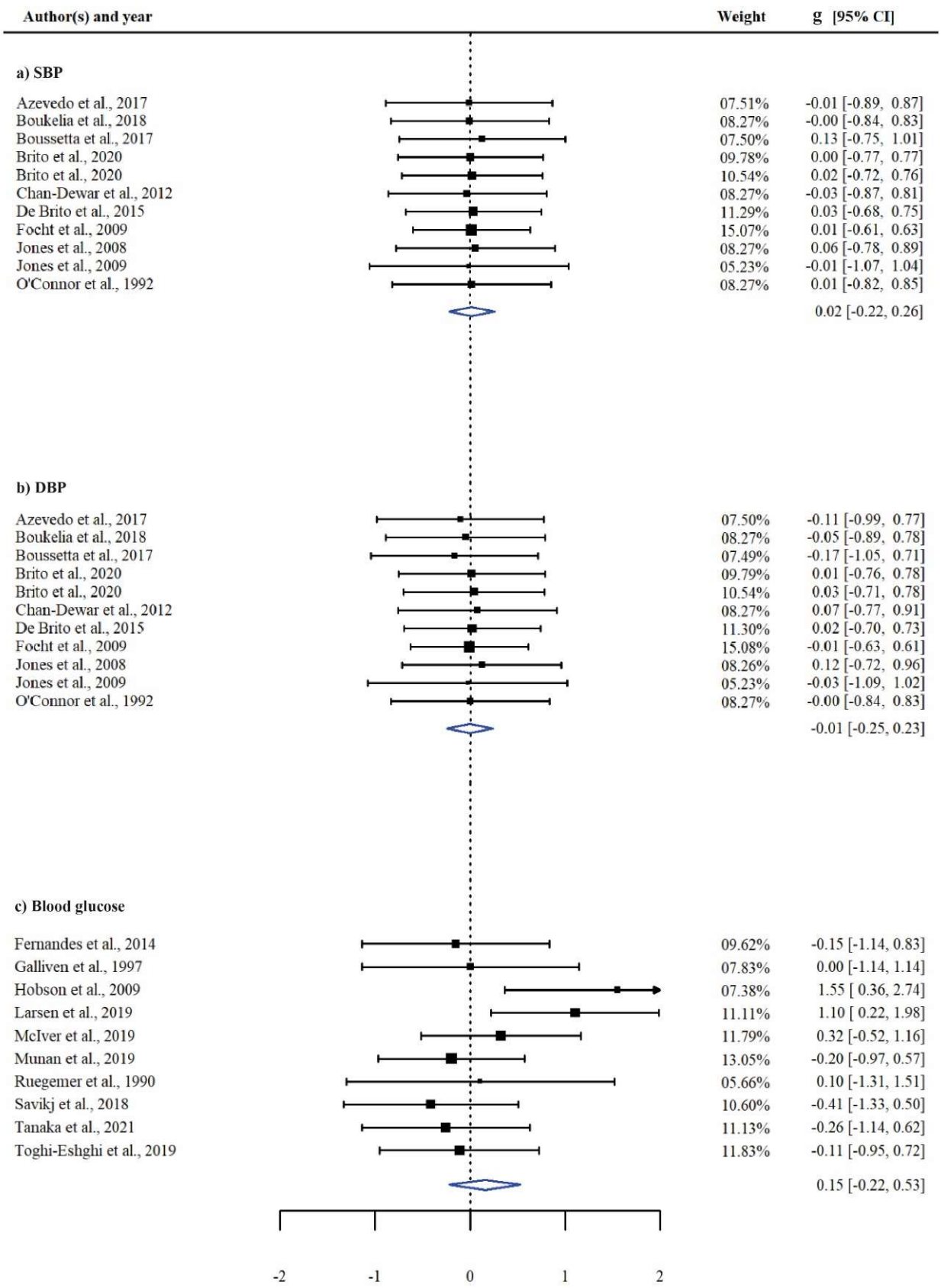


Figure 6. Forest plot of the standardized effect sizes (hedges' g) for a) systolic blood pressure (SBP), b) diastolic blood pressure (DBP) and c) blood glucose. A negative value means the effect of exercise performed in the evening is greater than the effect of exercise performed in the morning. CI = confident interval.

Table 4. Acute change in SBP, DBP and blood glucose after morning, evening, and morning vs. evening exercise

	Morning MD	Evening MD	Morning vs. Evening MD
	mmHg	mmHg	mmHg
SBP	-5.54 [-8.58, -2.49]	-6.43 [-9.29, -3.56]	1.43 [-1.47, 4.34]
DBP	-1.17 [-3.10, 0.76]	-0.92 [-2.16, 0.32]	0.02 [-1.37, 1.42]
	mmol/L	mmol/L	mmol/L
Blood glucose	0.43 [0.09, 0.77]	0.04 [-0.12, 0.19]	0.32 [-0.11, 0.76]

4.5. Risk of bias and quality assessment

The methodological quality of all trials included in the meta-analysis was assessed considering specific biases to cross-over design based on Cochrane's risk of bias tool[20]. According to these criteria, 47.5% of the total items were categorized as having a low, 44.9% unclear, and 7.6% high risk of bias. Details of this quality assessment can be found in Table 5. The analyses of this variable as a potential moderator of effect sizes revealed non-significant results ($P > 0.05$ for all groups and outcomes), so the methodological quality of studies did not affect effect sizes on cardiovascular risk factors.

4.6. Sensitivity analysis

Only 1 study was influencing exercise effect on SBP in the morning[41], 1 study was influencing exercise effect on MBP in the morning and evening[39] and 1 study was influencing exercise effect on blood glucose in the evening and morning vs. evening [44]. Yet, the reductions of pooled effects after excluding them from analyses were not significant ($\Delta g = \leq 0.14$), so we decided to include them in the total effect size calculation. Lastly, we found no risk of publication bias (fail-safe N index range from 0 to 100 Egger's test: $P > 0.05$).

Table 5. Risk of bias

	Domain 1: Randomization process			Domain S: Carryover effects		Domain 2: Deviation from intended intervention				Domain 3: Missing outcome data		Domain 4: Measuring outcomes		Domain 5: Report results	
	Item 1	Item 2	**RoB2**	Item 3	**RoB2**	Item 4	Item 5	Item 6	**RoB2**	Item 7	**RoB2**	Item 8	**RoB2**	Item 9	**RoB2**
Aldemir et al, 2005	Yes	No	🔴	Unclear	🟡	Yes	Unclear	Yes	🟢	Yes	🟢	No	🔴	Yes	🟢
Azevedo et al, 2017	Yes	Unclear	🟡	Unclear	🟡	Yes	Unclear	Yes	🟢	Unclear	🟡	No	🔴	Yes	🟢
Boukelia et al, 2018	Yes	Unclear	🟡	Unclear	🟡	Yes	Unclear	Yes	🟢	No	🔴	No	🔴	Unclear	🟡
Bousseta et al, 2017	No	Unclear	🔴	Unclear	🟡	Yes	Unclear	Yes	🟢	Yes	🟢	No	🔴	Yes	🟢
Brito et al, 2015	Yes	Yes	🟢	Unclear	🟡	Yes	Unclear	Yes	🟢	Yes	🟢	Yes	🟢	Yes	🟢
Brito et al, 2020	Yes	Yes	🟢	Unclear	🟡	Yes	Unclear	Unclear	🟡	Unclear	🟡	Unclear	🟡	Yes	🟢
Chan-Dewar et al, 2012	Unclear	Unclear	🟡	Unclear	🟡	Yes	Unclear	Yes	🟢	Yes	🟢	Unclear	🟡	Yes	🟢
Di Blasio et al, 2010	Yes	Unclear	🟡	Yes	🟢	Yes	Unclear	Unclear	🟡	Unclear	🟡	Unclear	🟡	Yes	🟢
Fernandes et al, 2014	Yes	Unclear	🟡	Unclear	🟡	Yes	Unclear	Yes	🟢	Yes	🟢	Yes	🟢	Yes	🟢
Focht et al, 2009	Yes	Unclear	🟡	Unclear	🟡	Yes	Unclear	Yes	🟢	Unclear	🟡	Unclear	🟡	Yes	🟢
Galliven et al, 1997	Yes	Unclear	🟡	Unclear	🟡	Yes	Unclear	Unclear	🟡	No	🔴	Unclear	🟡	Yes	🟢
Hobson et al, 2009	Yes	Yes	🟢	Unclear	🟡	Yes	Unclear	Yes	🟢	No	🔴	Yes	🟢	Yes	🟢

🟢 Low Risk 🟡 Some concerns 🔴 High risk

Table 5. Risk of bias

	Domain 1: Randomization process			Domain S: Carryover effects		Domain 2: Deviation from intended intervention				Domain 3: Missing outcome data		Domain 4: Measuring outcomes		Domain 5: Report results	
	Item 1	Item 2	**RoB2**	Item 3	**RoB2**	Item 4	Item 5	Item 6	**RoB2**	Item 7	**RoB2**	Item 8	**RoB2**	Item 9	**RoB2**
Jones et al, 2008	Yes	Unclear	🟡	Unclear	🟡	Yes	Unclear	Unclear	🟡	Unclear	🟡	Unclear	🟡	Yes	🟢
Jones et al, 2009	Yes	Unclear	🟡	Unclear	🟡	Yes	Unclear	Unclear	🟡	Unclear	🟡	Unclear	🟡	Yes	🟢
Larsen et al, 2019	Yes	Unclear	🟡	Unclear	🟡	Yes	Unclear	Yes	🟢	Yes	🟢	Unclear	🟡	Yes	🟢
McIver et al, 2019	Yes	Yes	🟢	Unclear	🟡	Yes	Unclear	Yes	🟢	Yes	🟢	Yes	🟢	Yes	🟢
Munan et al, 2020	Unclear	Yes	🟢	Unclear	🟡	Yes	Unclear	Yes	🟢	No	🔴	Yes	🟢	Yes	🟢
O'Connor et al, 1992	Unclear	Unclear	🟡	Unclear	🟡	Yes	Unclear	Unclear	🟡	Unclear	🟡	No	🔴	Yes	🟢
Ruegemer et al, 1990	Unclear	Unclear	🟡	Unclear	🟡	Yes	Unclear	Yes	🟢	Yes	🟢	Unclear	🟡	Yes	🟢
Savikj et al, 2018	Unclear	Yes	🟢	Unclear	🟡	Yes	No	Unclear	🔴	No	🔴	No	🔴	Yes	🟢
Tanaka et al, 2021	Yes	Unclear	🟡	Unclear	🟡	Yes	Unclear	Yes	🟢	Yes	🟢	Unclear	🟡	Yes	🟢
Toghi-Eshghi et al, 2019	Yes	Unclear	🟡	Unclear	🟡	Yes	Unclear	Unclear	🟡	No	🔴	Unclear	🟡	Yes	🟢

🟢 Low Risk 🟡 Some concerns 🔴 High risk

5. Discussion

We systematically reviewed and meta-analysed the results of 22 studies aiming to better understand whether the acute effect of exercise on cardiovascular disease risk factors in adults differs when it is performed in the morning vs. the evening. Overall, we found no influence of the time of the day (i.e., morning vs. evening) on the effect of a single bout of exercise on blood pressure neither on blood glucose.

The results of this systematic review and meta-analysis should however be taken with caution. Although a total of 22 studies have been included, we present 4 different meta-analyses each one corresponding to a different outcome: SBP (11 studies)[11–13,34–38,41,47], DBP (11 studies)[11–13,34–38,41,47], MBP (7 studies)[11–13,33,37,39] and blood glucose (10 studies)[10,40,42–46,48,49,51]. Further, qualitative differences between studies do exist (e.g., exercise protocols, health and training status, or sex). It should be noted as a limitation that the lack of statistical heterogeneity in any of the meta-analyses performed may be explained by the small sample size of included studies.

Exercise protocols consisted of aerobic training in most of the studies except for two of them, which used strength training[41,49], and a single study that used a mixed strength and aerobic training protocol[34]. Importantly, exercise intensity and duration differ between them. Also, some protocols were performed under hot and humid conditions[35,43]. We considered data measured between 15 to 60 minutes post-exercise, which is a relatively wide range in which results may differ. Likewise, not all exercise protocols were performed at the same hour but within a wide range of hours differing in each study (i.e., 6:00 to 9:30 a.m. for morning exercise and 3:00 to 9:00 pm for evening exercise). Blood glucose and blood pressure present diurnal rhythmicity themselves[53–55] and therefore their response to exercise may be different even within the same time slot. Of the 22 studies, only 6 included female participants[34,38,42,46,48,49]. We found few studies including trained participants [35,36,38,40] while most studies included sedentary or recreationally active people. About half of the studies included participants with normal-weight[12,33,47,35,36,38–43], the rest included participants with overweight and obesity[11,13,34,37,44–46,49,56]. Participants also differed in age, being the youngest sample of 21 years average[35] and the oldest of 65 years average[46]. A minor part of the total sample was composed of patients with pre-hypertension [13], hypertension[34,37], mild-sleep apnoea[44], type I diabetes[48,49], and type II diabetes[10,46], being the rest healthy participants. However, when analysing the moderator effects of exercise intensity and duration, hour within the morning and evening, sex, BMI, age, and health

status we found no influence of any of the variables regarding the effect of morning vs. evening exercise. The lack of significant moderator effects may be due to the reduced number of studies included in each moderator level and, in the case of sex, to the reduced number of women participating in the studies.

The results of this meta-analysis indicate that morning and evening exercise produces similar acute hypotensive effects. This is in line with most of the studies[33–36,38,39,41,47]. Discrepancies with other studies can be explained by health status (pre-hypertension)[13], medication[37], or inappropriate analysis (not taking into account baseline circadian rhythmicity)[11,12]. Albalak et al.[8] found an association between morning physical activity and lower risk of CVD in general population. Interestingly, their results were mostly driven by women (58%), which is a lacking population in our meta-analysis. Differences in the studies' design need also to be considered. In epidemiological studies[7–9] participants are organized into clusters according to the time of the day they are physically active, which is probably related to their individual circadian rhythmicity (i.e., chronotype). In contrast, in crossover studies each participant's chronotype may influence the response to morning and evening exercise. Sex and chronotype seem to be important when studying the effect of the time of the day of exercise.

In the present study, we did not find significant differences when comparing the acute effect of morning vs. evening exercise on blood glucose. However, the increase in blood glucose tended to be bigger after morning exercise. The rhythmicity of skeletal muscle's clock genes may explain these responses. Glucose uptake of the skeletal muscle cell dependent on the glucose transporter 4 is regulated by clock genes (specifically *CLOCK* and *ARNTL* genes[57]). Interestingly, in animal models, *CLOCK* has been described to peak during the light phase in retina cells and to peak later, during the dark phase, in the skeletal muscle[58]. Therefore, glucose uptake by the skeletal muscle cells (and consequently clearance of blood glucose) may be enhanced in the dark phase (i.e., in the evening hours). Although health status did not moderate the morning vs. evening response of blood glucose, in the separate meta-analyses of morning exercise we observed a significant increase in non-healthy participants (most with diabetes mellitus) but not in healthy. We speculate cortisol and insulin circadian rhythmicity are involved in the mentioned dissimilarity. Free cortisol and insulin levels are higher in the morning[40,59]. Cortisol is expected to produce an elevation in plasma glucose that results not notorious in healthy (because of the counteractive effect of insulin), but notorious in patients with type I or type II diabetes where insulin function is altered. Only studies that included participants

with diabetes mellitus reported significant time-of-day differences in blood glucose response to exercise: an increase after morning but not after evening exercise[10,48,49], except for Munan et al.[46], in which patients presented well-managed diabetes and were taking lowering-glucose medication (not an accurate representation of the average population with diabetes). Thus, it may well be that exercising in the morning should be avoided by patients with diabetes mellitus.

5.1. Future perspectives

There is no available evidence to clarify whether health status, age, BMI, type of exercise, and hour within the morning and evening influence the response to exercise at different times of the day. Similarly, we still need to elucidate whether this response is dependent on sex since studies including women are scarce. We neither found enough studies investigating blood lipids. Additionally, it would be of interest to investigate the acute response of other cardiovascular risks factors such as insulin or inflammatory markers.

We suggest some points to consider for future research. First, the resting diurnal rhythmicity present in blood pressure and blood glucose needs to be considered. Some studies highlight the importance of accounting for differences in initial values across conditions and the repeated measure design[38,47]. For example, when data from the studies that reported diurnal differences in the hypotensive acute effect of exercise[11–13,37] were analysed in this meta-analysis, we found very small effect sizes when comparing morning and evening exercise effects. We recommend controlling for chronotype, as performed in a few of the included studies[13,37,40,41,45,47]. It may also be interesting to control for peripheral temperature since changes in this factor can be attributed to alterations in circadian clocks[60].

6. Conclusion

The present preliminary findings reveal that exercise produces an acute reduction of systolic blood pressure independently of the time of the day at which it is performed. Similarly, exercise produces an acute increase in blood glucose independently of the time of the day. With the available literature, we could not make robust conclusions about the acute effect of the time of the day of exercise on cardiovascular disease risk factors. Further research is required to establish whether there is a diurnal variation of exercise on cardiovascular health and how it is related to health status, sex, or the type of exercise.

7. References

1. Mure LS, Le HD, Benegiamo G, et al. Diurnal transcriptome atlas of a primate across major neural and peripheral tissues. Science (80-) 2018; 359(6381).

2. Gabriel BM, Zierath JR. Circadian rhythms and exercise — re-setting the clock in metabolic disease. Nat Rev Endocrinol 2019:197–206.

3. Dyar KA, Ciciliot S, Wright LE, et al. Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock. Mol Metab 2014; 3(1):29–41.

4. Vetter C, Dashti HS, Lane JM, et al. Night shift work, genetic risk, and type 2 diabetes in the UK biobank. Diabetes Care 2018; 41(4):762–769.

5. Vera B, Dashti HS, Gómez-Abellán P, et al. Modifiable lifestyle behaviors, but not a genetic risk score, associate with metabolic syndrome in evening chronotypes. Sci Rep 2018; 8(1).

6. Kraus WE, Powell KE, Haskell WL, et al. Physical Activity, All-Cause and Cardiovascular Mortality, and Cardiovascular Disease. Med Sci Sports Exerc 2019; 51(6):1270–1281.

7. Albalak G, Stijntjes M, Wijsman CA, et al. Timing of objectively-collected physical activity in relation to body weight and metabolic health in sedentary older people: a cross-sectional and prospective analysis. Int J Obes (Lond) 2022; 46(3):515–522.

8. Albalak G, Stijntjes M, Van Bodegom D, et al. Setting your clock: associations between timing of objective physical activity and cardiovascular disease risk in the general population. Eur J Prev Cardiol 2022; 00:1–9.

9. van der Velde JHPM, Boone SC, Winters-van Eekelen E, et al. Timing of physical activity in relation to liver fat content and insulin resistance. Diabetologia 2022.

10. Savikj M, Gabriel BM, Alm PS, et al. Afternoon exercise is more efficacious than morning exercise at improving blood glucose levels in individuals with type 2 diabetes: a randomised crossover trial. Diabetologia 2019; 62(2):233–237.

11. Jones H, Taylor CE, Lewis NCS, et al. Post-exercise blood pressure reduction is greater following intermittent than continuous exercise and is influenced less by diurnal variation. Chronobiol Int 2009; 26(2):293–306.

12. Jones H, Pritchard C, George K, et al. The acute post-exercise response of blood pressure varies with time of day. Eur J Appl Physiol 2008; 104(3):481–489.

13. De Brito LC, Rezende RA, Da Silva ND, et al. Post-exercise hypotension and its mechanisms differ after morning and evening exercise: A randomized crossover study. PLoS One 2015; 10(7):1–16.

14. Page MJ, McKenzie JE, Bossuyt PM, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021; 372.

15. Botella J, Gambara H. Doing and reporting a meta-analysis. © Int J Clin Heal Psychol 2006; 6(2):425–440.

16. Carneiro-Barrera A, Díaz-Román A, Guillén-Riquelme A, et al. Weight loss and lifestyle interventions for obstructive sleep apnoea in adults: Systematic review and meta-analysis. Obes Rev 2019; 20(5):750–762.

17. Carneiro-Barrera A, Amaro-Gahete FJ, Buela-Casal G. Selecting studies (not reports) as the unit of interest of systematic reviews and meta-analyses: an essential practice. Int J Obes 2020 447 2020; 44(7):1536–1538.

18. Carpio-Rivera E, Moncada-Jiménez J, Salazar-Rojas W, et al. Acute Effects of Exercise on Blood Pressure: A Meta-Analytic Investigation. Arq Bras Cardiol 2016; 106(5):422.

19. Colberg SR, Hernandez MJ, Shahzad F. Observations Blood Glucose Responses to Type, Intensity, Duration, and Timing of Exercise. Diabetes Care. 2013;36(10):e177.

20. Higgins JPT, Eldridge S, Li T. Including variants on randomized trials. Cochrane Handb Syst Rev Interv 2019:569–593.

21. Kebede MM, Peters M, Heise TL, et al. Comparison of three meta-analytic methods using data from digital interventions on type 2 diabetes. Diabetes, Metab Syndr Obes Targets Ther 2019; 12:59.

22. Lakens D. Calculating and reporting effect sizes to facilitate cumulative science: a practical primer for t-tests and ANOVAs. Front Psychol 2013; 0:863.

23. Madeyski L, Kitchenham B. Effect sizes and their variance for AB/BA crossover design studies. Empir Softw Eng 2018; 23(4):1982–2017.

24. Goulet-Pelletier J-C, Cousineau D. A review of effect sizes and their confidence intervals, Part I: The Cohen's d family. Quant Methods Psychol 2018; 14(4):242–265.

25. Nakagawa S, Cuthill IC. Effect size, confidence interval and statistical significance: a practical guide for biologists. Biol Rev Camb Philos Soc 2007; 82(4):591–605.

26. Higgins JPT, Thompson SG. Quantifying heterogeneity in a meta-analysis. Stat Med 2002; 21(11):1539–1558.

27. Sauseng-Fellegger G, Konig EM, Hinghofer-Szalkay H, et al. Testing of neuroendocrine function in astronauts as related to fluid shifts. Acta Astronaut 1992; 27:55–60.

28. Park S, Jastremski CA, Wallace JP. Time of day for exercise on blood pressure reduction in dipping and nondipping hypertension. J Hum Hypertens 2005; 19(8):597–605.

29. Jones H, George K, Edwards B, et al. Effects of time of day on post-exercise blood pressure: Circadian or sleep-related influences? Chronobiol Int 2008; 25(6):987–998.

30. Gomez AM, Gomez C, Aschner P, et al. Effects of performing morning versus afternoon exercise on glycemic control and hypoglycemia frequency in type 1 diabetes patients on sensor-augmented insulin pump therapy. J Diabetes Sci Technol 2015; 9(3):619–624.

31. Cardoso SS, Feuers RJ, Tsai TH, et al. Chronobiology of exercise: the influence of scheduling upon glycemic responses of control and of subjects with diabetes mellitus. Prog Clin Biol Res 1990; 341B(53):345–353.

32. All in good time. Does the time of day you exercise matter in blood glucose management? Diabetes Forecast 2015; 68(2):19–20.

33. Hatice, Aldemir NK. The effect of time of day and exercise on platelet functions and platelet – neutrophil aggregates in healthy male subjects Hatice Aldemir and Nedret Kılı ̧. Mol Cell Biochem 2005; 280(1–2):119–124.

34. Azevêdo LM, Souza AC de, Santos LES, et al. Fractionated Concurrent Exercise throughout the Day Does Not Promote Acute Blood Pressure Benefits in Hypertensive Middle-aged Women. Front Cardiovasc Med 2017; 4:1–7.

35. Boukelia B, Gomes EC, Florida-James GD. Diurnal variation in physiological and immune responses to endurance sport in highly trained runners in a hot and humid environment. Oxid Med Cell Longev 2018; 2018.

36. Boussetta N, Abedelmalek S, Aloui K, et al. The effect of air pollution on diurnal variation of performance in anaerobic tests, cardiovascular and hematological parameters, and blood gases on soccer players following the Yo–Yo Intermittent Recovery Test Level-1. Chronobiol Int 2017; 34(7):903–920.

37. Brito LC, Azevêdo L, Peçanha T, et al. Effects of ACEi and ARB on post-exercise hypotension induced by exercises conducted at different times of day in hypertensive men. Clin Exp Hypertens 2020; 42(8):722–727.

38. Chan-Dewar F, Gregson W, Whyte G, et al. Do the effects of high intensity 40 km cycling upon left ventricular function and cardiac biomarker during recovery vary with time of day? J Sports Sci 2013; 31(4):414–423.

39. Di Blasio A, D'Angelo E, Gallina S, et al. Relationship between time of day and variation of blood pressure response to aerobic exercise. Med Sport (Roma) 2010; 63(2):167.

40. Fernandes AL, Lopes-Silva JP, Bertuzzi R, et al. Effect of time of day on performance, hormonal and metabolic response during a 1000-M cycling time trial. PLoS One 2014; 9(10).

41. Focht BC, Koltyn KF. Alterations in pain perception after resistance exercise performed in the morning and evening 2009; 23(3):891–897.

42. Galliven EA, Singh A, Michelson D, et al. Hormonal and metabolic responses to exercise across time of day and menstrual cycle phase. J Appl Physiol 1997; 83(6):1822–1831.

43. Hobson RM, Clapp EL, Watson P, et al. Exercise capacity in the heat is greater in the morning than in the evening in man. Med Sci Sports Exerc 2009; 41(1):174–180.

44. Larsen P, Marino F, Melehan K, et al. Evening high-intensity interval exercise does not disrupt sleep or alter energy intake despite changes in acylated ghrelin in middle-aged men. Exp Physiol 2019; 104(6):826–836.

45. McIver VJ, Mattin LR, Evans GH, et al. Diurnal influences of fasted and non-fasted brisk walking on gastric emptying rate, metabolic responses, and appetite in healthy males. Appetite 2019; 143:104411.

46. Munan M, Dyck RA, Houlder S, et al. Does exercise timing affect 24-hour glucose concentrations in adults with type 2 diabetes? A follow up to the Exercise-Physical Activity and Diabetes glucose Monitoring Study. Can J Diabetes 2020; 44(8):711–718.

47. O'Connor PJ, Davis JC. Psychobiologic responses to exercise at different times of day. Med Sci Sports Exerc 1992:714–719.

48. Ruegemer, J., Squieres, Ray W., Marsh, Harold M., Haymond, Morey W., Cryer, Philip E., Rizza, Robert A., Miles JM. Differences Between Prebreakfast and Late Afternoon Lgycemic Responses to Exercise in IDDM Patients. J Chem Inf Model 2019; 53(9):1689–1699.

49. Toghi-Eshghi SR, Yardley JE. Morning (Fasting) vs Afternoon Resistance Exercise in Individuals with Type 1 Diabetes: A Randomized Crossover Study. J Clin Endocrinol Metab 2019; 104(11):5217–5224.

50. Tanaka Y, Ogata H, Park I, et al. Effect of a single bout of morning or afternoon exercise on glucose. Physiol Soc 2021; 9(7):e14784.

51. Tanaka Y, Ogata H, Park I, et al. Effect of a single bout of morning or afternoon exercise on glucose fluctuation in young healthy men. Physiol Rep 2021; 9(7).

52. Aloui K, Abedelmalek S, Chtourou H, et al. Effects of time-of-day on oxidative stress, cardiovascular parameters, biochemical markers, and hormonal response following level-1 Yo-Yo intermittent recovery test. Physiol Int 2017; 104(1):77–90.

53. Millar-Craig MW, Bishop CN, Raftery EB. Circadian variation of blood-pressure. Lancet 1978; 311(8068):795–797.

54. Rudic RD, McNamara P, Curtis AM, et al. BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. PLoS Biol 2004; 2(11).

55. Feneberg R, Sparber M, Veldhuis JD, et al. Synchronous fluctuations of blood insulin and lactate concentrations in humans 1. J Clin Endocrinol Metab 1999; 84(1):220–227.

56. Frandsen J, Pistoljevic N, Quesada JP, et al. Menstrual cycle phase do not affect whole-body peak fat oxidation rate during exercise. J Appl Physiol 2020;128(3):681-687. 57. Stenvers DJ, Scheer FAJL, Schrauwen P, et al. Circadian clocks and insulin resistance. Nat Rev Endocrinol 2019; 15(2):75–89.

58. Ceinos RM, Chivite M, López-Patiño MA, et al. Differential circadian and light-driven rhythmicity of clock gene expression and behaviour in the turbot, Scophthalmus maximus. PLoS One 2019; 14(7):e0219153.

59. Teo W, Newton MJ, McGuigan MR. Circadian Rhythms in Exercise Performance: Implications for Hormonal and Muscular Adaptation. J Sports Sci Med 2011; 10(4):600.

60. Brown SA, Zumbrunn G, Fleury-Olela F, et al. Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Curr Biol 2002; 12(18):1574–1583.

Supplementary material for Chapter 3: Time of the day of exercise impact on cardiovascular disease risk factors in adults: a systematic review and meta-analysis.

Table S1. PRISMA checklist.

Section and topic	Item #	Checklist item	Location where item is reported
Title			
Title	1	Identify the report as a systematic review.	Title
Abstract			
Abstract	2	See the PRISMA 2020 for Abstracts checklist (table 2).	Abstract
Introduction			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	43-44
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	44
Methods			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	44-45

Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	45
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Table 1
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	45-46
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	45-46

Data items	10[a]	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	44
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	45-46
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	46
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	47-48
Synthesis methods	13[a]	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	45-46

	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	47-48
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	46
	13d	Describe any methods used to synthesise results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	47
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	47
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesised results.	47
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	46
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	47

Results			
Study selection	16[a]	Describe the results of the search and selection process, from the number of records identified in the search to the nurmber of studies included in the review, ideally using a flow diagram (see Figure 5).	Figure 5
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	50
Study characteristics	17	Cite each included study and present its characteristics.	50
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Table 5
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Figure S1 – S4
Results of syntheses	20[a]	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Table 5
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible	56

		interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	56
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesised results.	56
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	58
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	58
Discussion			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	61
	23b	Discuss any limitations of the evidence included in the review.	61-63
	23c	Discuss any limitations of the review processes used.	61-63
	23d	Discuss implications of the results for practice, policy, and future research.	63

Other information			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	44
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	44
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	44
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	8
Competing interests	26	Declare any competing interests of review authors.	8
Availability of data, code, and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	8

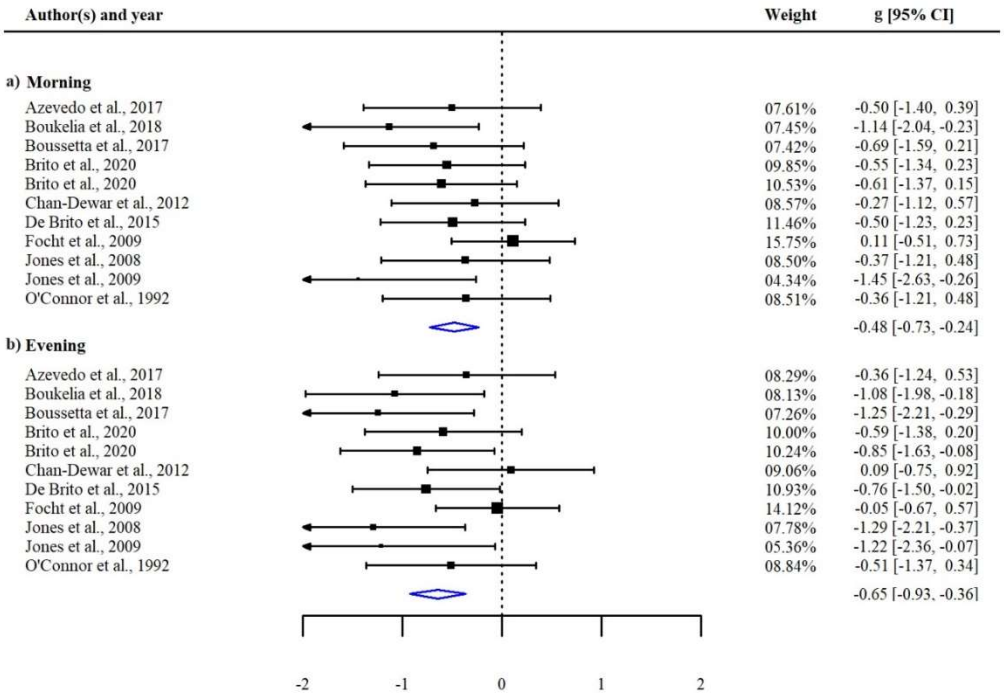


Figure S1. Forest plot of the standardized effect sizes (hedges' g) for systolic blood pressure (SBP) grouped by: a) effect of morning exercise (pre vs. post) and b) effect of evening exercise (pre vs. post). A negative value means an acute reduction of the outcome 15 – 60 minutes after exercise. CI = confident interval

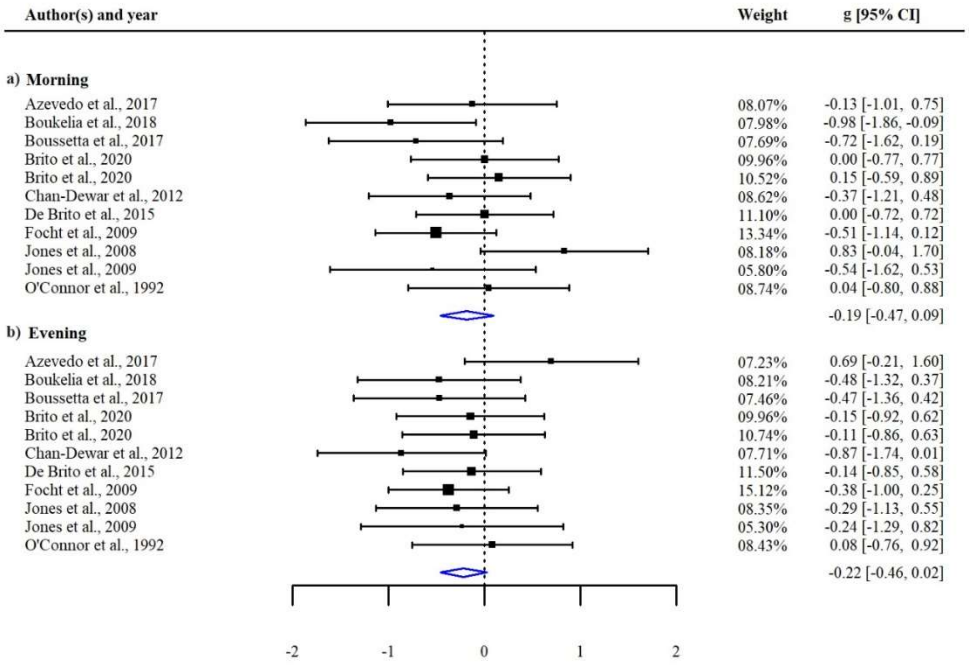


Figure S2. Forest plot of the standardized effect sizes (hedges' g) for diastolic blood pressure (DBP) grouped by: a) effect of morning exercise (pre vs. post) and b) effect of evening exercise (pre vs. post). A negative value means an acute reduction of the outcome 15 – 60 minutes after exercise. CI = confident interval

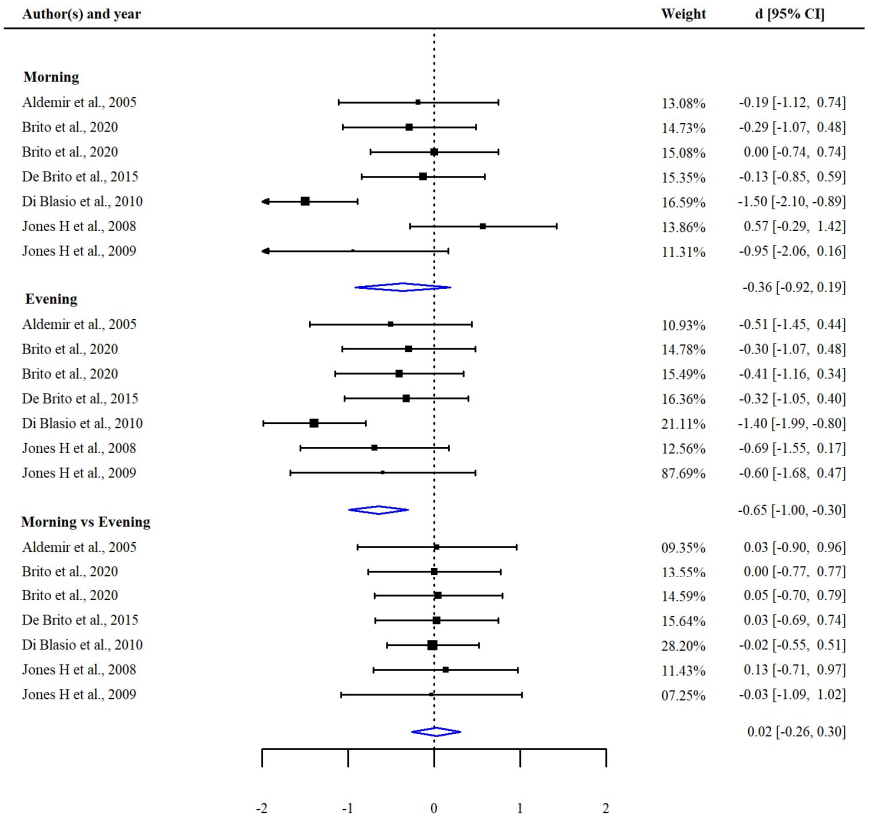


Figure S3. Forest plot of the standardized effect sizes (hedges' g) for mean blood pressure (MBP) grouped by: a) effect of morning exercise (pre vs. post), b) effect of evening exercise (pre vs. post), and c) effect of the time of the day (morning vs. evening effects). For a) and b), a negative value means an acute reduction of the outcome 15 – 60 minutes after exercise. For c), a negative value means the effect of exercise performed in the evening is greater than the effect of exercise performed in the morning. CI = confident interval

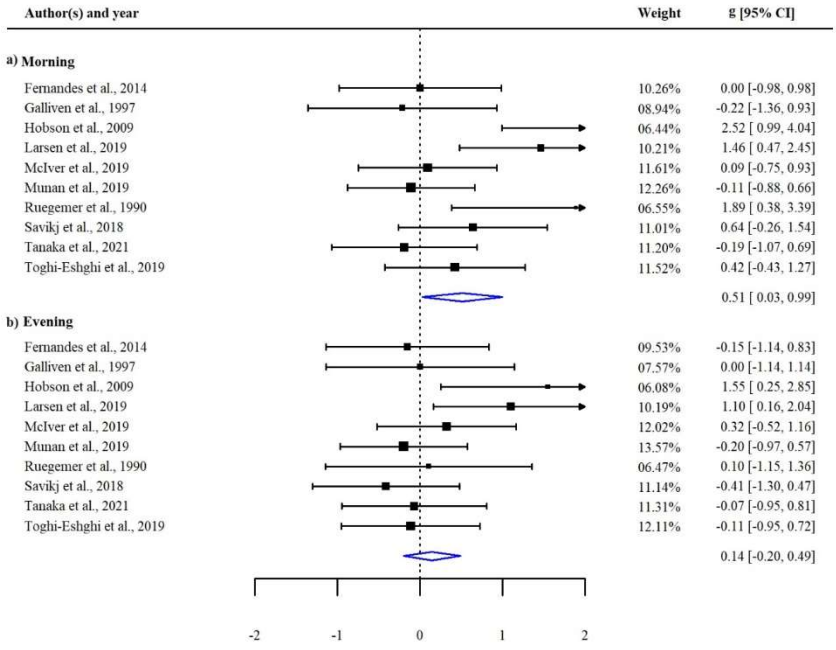
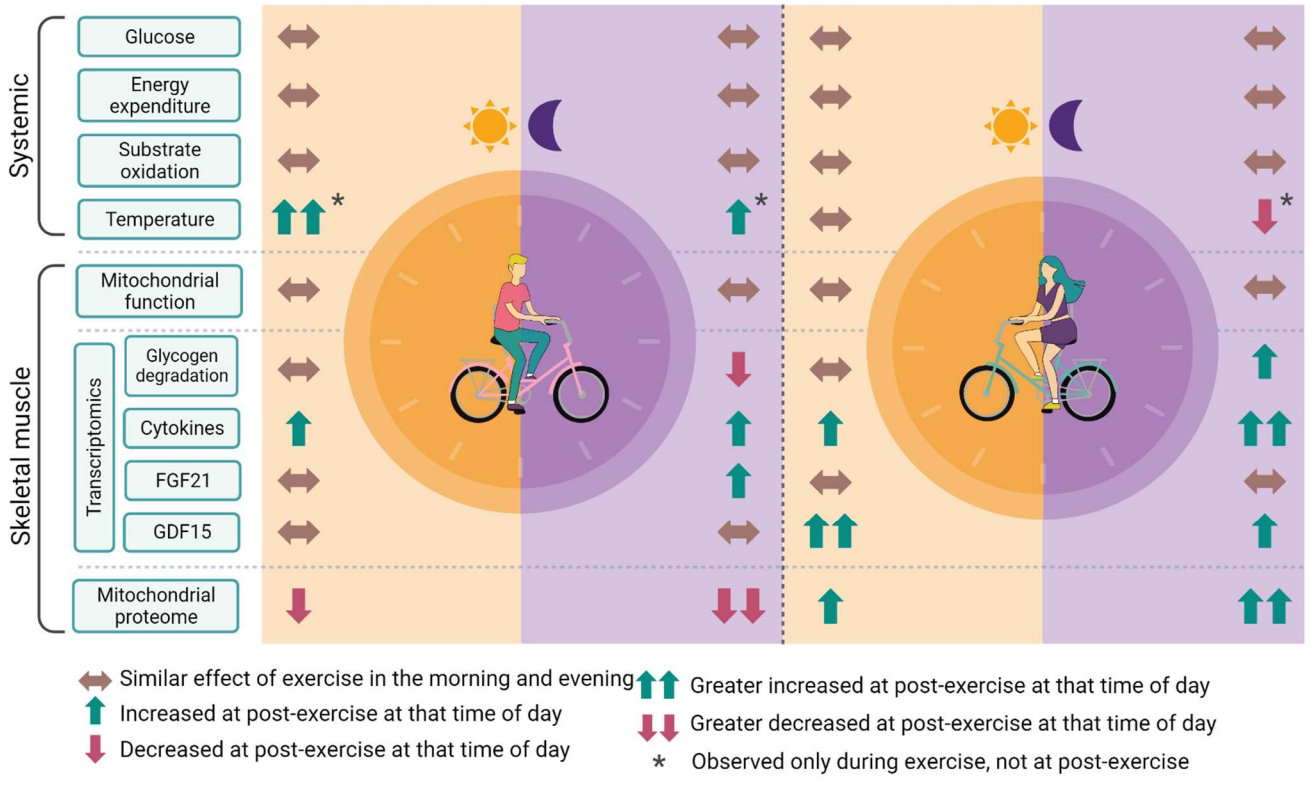


Figure S4. Forest plot of the standardized effect sizes (hedges' g) for diastolic blood glucose grouped by: a) effect of morning exercise (pre vs. post) and b) effect of evening exercise (pre vs. post). A negative value means an acute reduction of the outcome 15 – 60 minutes after exercise. CI = confident interval

Chapter 4. Sexual dimorphism on the acute effect of exercise in the morning vs. evening: A Randomized Crossover Study

1. Abstract

Mammalian cells possess molecular clocks which adequate functioning is decisive for metabolic health. Exercise is known to modulate these clocks, potentially having distinct effects on metabolism depending on the time of day. Using a randomized crossover design, this study investigated the impact of morning vs. evening moderate-intensity aerobic exercise on glucose regulation (continuous glucose monitoring) and energy metabolism in healthy men and women. It also aimed to elucidate molecular mechanisms within skeletal muscle. Results indicated similar systemic glucose and energy metabolism during and after exercise in both sexes. Notably, transcriptional analysis, mitochondrial function, and mitochondrial proteomics revealed marked sexual dimorphism and time of day variations. These findings may translate into observable systemic effects with higher exercise-intensity or chronic exercise interventions and provides a foundational molecular framework for precise exercise prescription in clinical setting.



Graphical Abstract

2. Introduction

Mammalian cells and tissues possess molecular clocks that coordinate physiology working as a coupled network [1]. Cues such as light/dark, food and physical activity interact with the molecular clocks to generate approximately 24-hour oscillations (i.e., circadian rhythm) in the function of thousands of genes[1]. Adequate functioning of this circadian machinery is decisive for optimal metabolic health[2]. In humans, its disruption through shifted sleep patterns or inadequate eating window has demonstrated to increase the risk of obesity, type 2 diabetes mellitus and cardiovascular diseases[2].

Exercise is a powerful and well-recognized modulator of the skeletal muscle metabolism[3]. Indeed, aligning the timing of exercise (i.e., the moment of the day when exercise is performed) with the individual's circadian rhythms could be an effective approach in maximizing the positive influence it has on metabolic function and overall health[4]. Epidemiological studies underscore the relevance of the time of exercise for inducing health benefits[5–7], but the results are not conclusive regarding glucose homeostasis[8] or lipid metabolism[9]. Some studies have reported that fat oxidation[10–12] and energy expenditure (EE)[11] were higher in the evening, whereas others did not observe any discernible differences in these outcomes based on the time of day (morning vs. evening)[13–17]. Crucially, the majority of these studies have been focused on men. Women remain disproportionately underrepresented in exercise medicine research despite significant biological differences[18]. Given the need of action to redress this sex gap in research and the growing interest surrounding the comprehension of women's physiology over recent decades, there exists a noteworthy scientific, clinical, and practical interest in ascertaining the molecular and physiological mechanisms explaining the potential exercise-induced diurnal variation differences between sexes on health-related metabolic parameters.

This study aimed to determine the differential impact of an acute exercise session performed in the morning vs. evening on glucose regulation, energy metabolism and metabolic health in healthy men and women, and to explore the underlying skeletal muscle molecular mechanisms. Investigating the optimal time for exercise is a matter of clinical and public health importance in the prevention and treatment of metabolic diseases. Exploring distinct responses in both men and women is imperative for addressing sex gap and creating inclusive guidelines. Further, examining molecular mechanisms can be of interest for implementing precision medicine in the clinical setting.

3. Methods

3.1. Subject details

Thirty-five healthy adults (men, n=18; women, n=17) completed the current trial. Participants mostly presented intermediate and moderate chronotypes. Recruitment was done via advertisements in social networks and at different faculties of the University of Granada (Spain). Inclusion criteria were i) age between 18 and 50 years, ii) body mass index (BMI): 18.5-27 kg/m^2 and iii) practising exercise less than 5 days per week. Exclusion criteria were i) history of disease (i.e., major adverse cardiovascular event, kidney failure, cirrhosis, eating disorder, weight control surgery, HIV/AIDS, rheumatoid arthritis, Parkinson's disease, active cancer treatment in the past year, diabetes mellitus), ii) use of drugs or medications that may affect the results, iii) unstable body weight for 3 months before the start of the study (> 4 kg weight loss or gain), iv) pregnancy and breastfeeding, v) active tobacco abuse or illicit drug use or a history of alcohol abuse treatment, vi) on a special diet or prescribed for other reasons (e.g., celiac disease). Sample size was calculated based on the results of a previous pilot study conducted in our laboratory. A total of 17 participants are needed to observe statistically significant differences between conditions (morning vs. evening) in fat oxidation during exercise [~10-15%] (80% statistical power and an α of 0.05). In order to conduct the analyses in men and women separately, we finally recruited 18 men and 17 women.

3.2. Study design

The present study is a randomised crossover trial. In a randomised and counterbalanced order, each participant performed a single bout of aerobic exercise at two different times of day: (i) morning (i.e., 11:30 a.m.), and (ii) evening (i.e., 6:30 p.m.) with a washout period of at least 3 days in between. This trial strictly followed CONSORT guidelines (http://www.consort-statement.org/consort-statement) and was registered at the https://clinicaltrials.gov (NCT05369715).

3.3. Study approval

All procedures were performed according to the Declaration of Helsinki, and it was approved by the Human Research Ethics Committee of the University of Granada and the Provincial Human Research Ethics Committee (Junta de Andalucía, ref. 1288-N-20). All

participants gave their oral and written informed consent. Data for each participant were collected over 4 visits. Figure 7 shows the overall design of the study.

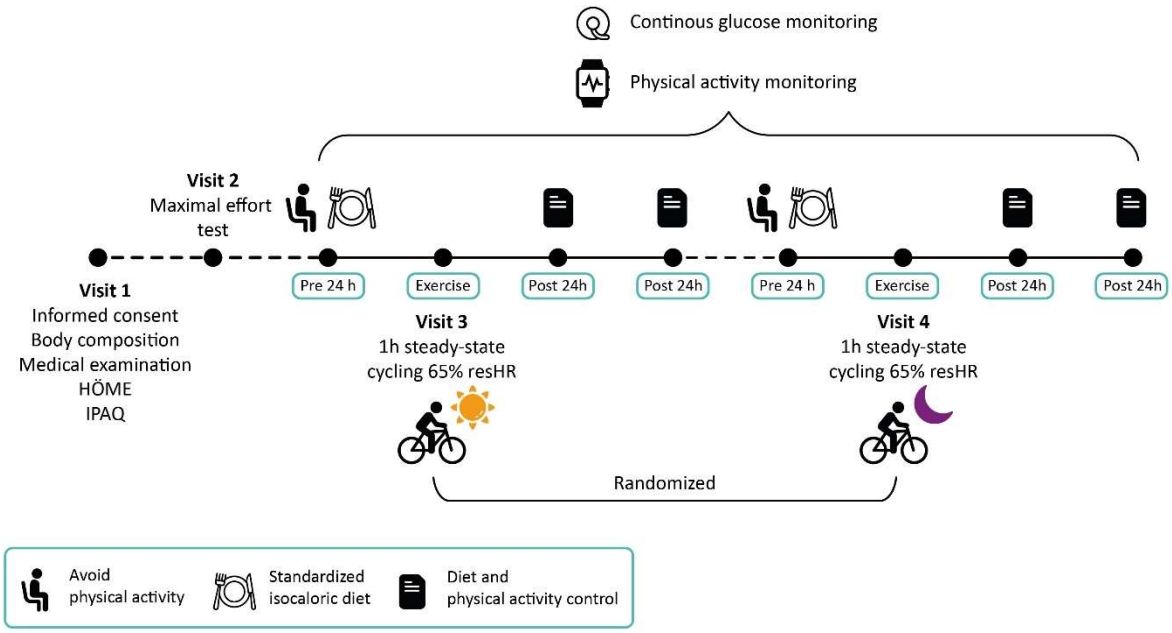


Figure 7. Overall procedures used in the study. Visit 1: preliminary examinations and body composition; visit 2: maximal effort test and start monitoring interstitial glucose (Continuous Glucose monitor) and physical activity (accelerometer); visits 3 and 4: one hour steady state test on cycle-ergometer at 65% resHR randomly at 11:30 or 18:30. Every exercise protocol was separated by at least 3 days. Participants were asked to follow and standardized isocaloric diet and refrain from moderate and vigorous physical activity the previous day. The first and second day after visit 3 participants registered every meal and physical activity and replicate it after visit 4. Each black circle represents one day. Continuous lines connect consecutive days, dashed lines connect days that may not be consecutive. Abbreviations: HÖME, HÖME Morningness–Eveningness Questionnaire; IPAQ, International Physical Activity Questionaire; resHR, Heart Rate Reserve.

3.4. Visit 1. Preliminary examination

Sociodemographic, lifestyle and body composition data were registered, and a medical screening was performed. Participants completed the HÖME questionnaire to determine individual's chronotype (i.e., morningness–eveningness), and the short version of the International Physical Activity Questionnaire (IPAQ) to assess their physical activity levels. Height and weight were measured (no shoes, light clothing) using a model 799 Seca scale and stadiometer (Seca, Hamburg, Germany). Body fat mass and percentage, lean body mass, and visceral adipose tissue (VAT) mass were evaluated by dual X-ray absorptiometry (Discovery Wi, Hologic, Inc, Bedford, MA, USA) and analysed by APEX software. The device was calibrated each day using a lumbar spine phantom. Participants

were asked to remain still while being scanned in the supine position, as per guidelines from the International Society of Clinical Densitometry[19]. BMI, lean mass, and fat mass indexes were expressed as kg/m^2.

3.5. Visit 2. Cardiorespiratory fitness

Cardiorespiratory fitness was assessed through a maximal effort test in an Ergoselect 200 cycle ergometer (Ergoline GmbH, Lindenstrasse, Germany). Previous physical activity and diet were controlled (see: "Control of cofounder variables"). A Polar OH1 photoplethysmographic heart-rate monitor (Polar Electro Oy, Kempele, Finland) was placed in the forearm to measure heart rate. After a resting period of 10 minutes, the maximum effort test started with a 3 min stage at 20 watts (W) as a warm-up, followed by increments of 20 W every 3 min until the respiratory exchange ratio (RER) was ≥1 at least for 30s [20]. At this point, further increments of 20 W every 1 min were implemented (with no interruptions) until (i) volitional exhaustion was reached, or (ii) participants had to stop because of peripheral fatigue. Through the maximum effort test, participants' fatigue perception was assessed using a rating of perceived exertion (RPE) scale (Borg CR10 Scale ®). Respiratory gas exchange was measured during the entire exercise test by indirect calorimetry (Quark CPET, Cosmed, Rome, Italy) and collected with a facemask (Hans Rudolph, Inc., Shawnee, KS, USA). According to the manufacturer's recommendations, volume was calibrated using a 3 L calibration syringe and the gas analyzer was calibrated using standard gas concentrations (O_2 = 16%, CO_2 = 5%) immediately before each trial. Resting and maximum heart rate were used to calculate exercise' intensity in the following visits. Gas exchange data were exported from the metabolic cart to an Excel spreadsheet in a sample frequency of 5 sec. Maximal volume of oxygen consumption (VO_2 max) was defined as a respiratory exchange ratio of ≥1.1, once a VO_2 plateau was reached and having attained a heart rate value within 10 beats/min of the individuals' age- predicted maximum (209-0.73 × age)[21] during the maximal effort exercise test. When participants did not achieve the VO_2 max criteria, VO_2 peak was used as the highest VO_2 value that was not an artifact (we screened the data set from the 2nd to the 10th subsequent largest VO2 uptake value). This value was calculated relative to the body mass.

Control of cofounding variables (visit 2)

Participants were asked to complete the following pre-experimental conditions: (i) to refrain vigorous physical activity the previous 48 hours and moderate physical activity the

previous 24 hours, (ii) to avoid caffeine ingestion 12 hours before, and (iii) to sleep with normality the night before. In case of women, the phase of their menstrual cycle was registered. Room temperature was controlled to maintain a range between 20-24ºC. Participants attended the laboratory in fasting conditions (4 h) after the ingestion of a complete self-selected meal.

3.6. Visits 3 and 4. Morning and evening moderate-intensity aerobic exercise

On third and fourth visits (Figure 8), participants performed a 60 min steady-state exercise bout on the same cycle ergometer used for the effort test at an intensity of 65% of their heart rate reserve (HRR) either at 11:30 a.m. or 6:30 p.m. The conditions (morning or evening, respectively) were randomized, and visits were separated by a range of 4 to 25 days (median = 7).

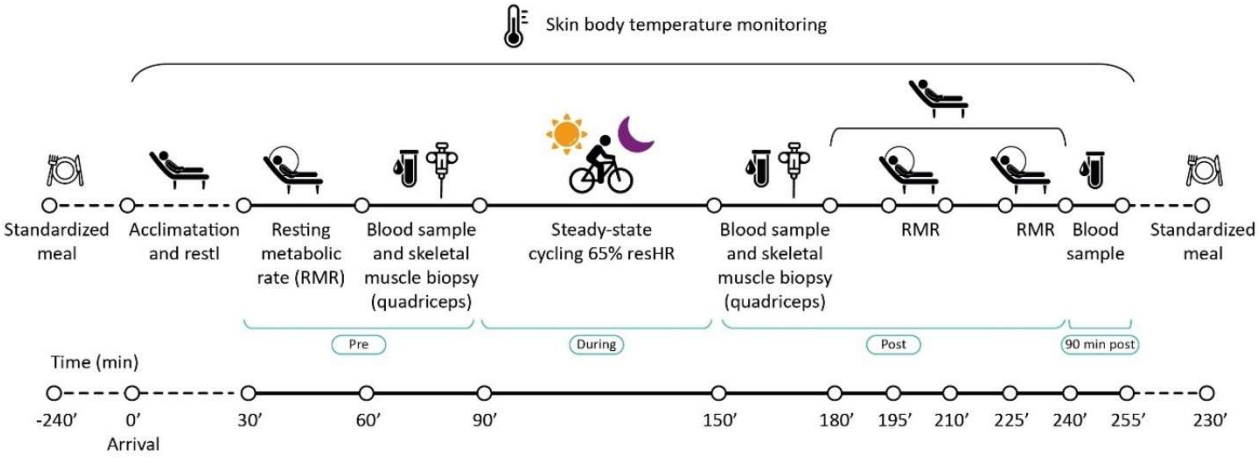


Figure 8. Detailed procedures on visits 3 and 4, where exercise (time point: 90') was performed either at 11:30 or 18:30 in random order. A standardized meal (i.e.: bread, cheese, olive oil and fruit) was consumed 4 hours before arrival (-240'). At arrival (0') a heart rate monitor was placed in the forearm and 16 thermal iButton™. were attached to different skin points. After an acclimatation period, gas exchange at rest was measured (30') and a first blood sample was collected (60'). Then, gas exchange was measured during 60 minutes of steady state exercise at 65% resHR (90'). A second blood sample after the test (150') was collected. They lay down again to stay 60 minutes resting where gas exchange was measured for 30 minutes in two separated 15 minutes stages (195' and 225'). To finish, a third blood sample was completed (240'). Participants were asked to consume a standardized meal within 1 hour after leaving the laboratory (i.e.: salad with lettuce, tomato, sweet corn, carrot, tuna and olive oil, and potato omelette with egg, potato, and olive oil). A skeletal muscle micro-biopsy was obtained from a subsample of 6 men and 8 women before and after exercise (60' and 150'). Abbreviations: RMR, resting metabolic rate; resHR, Heart Rate Reserve.

Continuous glucose monitoring

At least 24 hours before the third visit, participants started to wear a continuous glucose monitor (FreeStyle LibrePro, Abbot, Alameda, CA, USA) in the non-dominant upper-arm and maintained until the end of the study. We calculated hourly values and defined daytime interstitial glucose levels as the mean glucose concentration from 06:00 hours to 23:59 hours and nocturnal glucose as the mean glucose concentration from 24:00 hours to 05:59 hours as recommended by current guidelines[22]. For both morning and evening conditions, mean, area under the curve (AUC) and coefficient of variation (CV) were calculated for the 24 hours previous to exercise day (i.e., Pre 24h), the 24 hours of the exercise day (i.e., Exercise), and the two subsequent days to exercise (i.e., Post 24 h and Post 48 h, respectively) (Figure 7).

Physical activity and sleep

At least 24 hours before the third visit, participants were fitted with a non-dominant wrist-worn triaxial accelerometer (ActiGraph GT3X+, Pensacola, Florida, USA) that they wore until the end of the study. The devices were initialized to collect raw accelerations at a frequency of 100 Hz for at least seven consecutive days (24 hours/day). In addition, participants were instructed to register the bedtime and the wake-up time every day. Raw data from the accelerometers were downloaded using the ActiLife software (ActiGraph, Pensacola, FL, USA) and then processed with the open-source GGIR R package, version 3.0-0 [23]. In brief, the Euclidean Norm of the raw accelerations Minus One *G* with negative values rounded to zero (ENMO) was calculated over 5-second epochs; non-wear time periods were identified from the magnitude and variability of the raw accelerations measured at each accelerometer axis[24], and imputed when appropriate by the average ENMO at the same time interval during the rest of the recording days; sleep and awake periods were identified using an automated algorithm based on the variability of the arm posture and guided by the sleep times reported by the participants. Then, sedentary (<30 m*g*), light (30-99 m*g*), and moderate-to-vigorous (≥ 100 m*g*) activities were classified using the Hildebrand et al. cut-points [25,26]. Finally, moderate-to-vigorous physical activity was considered when the activity above the moderate-to-vigorous threshold lasted at least 5 minutes, allowing for a maximum of 1 minute below the threshold. During the sleep period time, minute-by-minute classification of sleep vs awake status was conducted, and then total sleep time, total time awake after sleep onset[27], and sleep efficiency were calculated for analytical purposes.

Energy metabolism assessment

Resting period before exercise

Participants arrived the laboratory (Figure 8, time point: 0') and lay down in a stretcher and stay relaxed for 30 min (acclimatation period), a time during which they were instructed not to move, talk, sleep nor cross their arms and/or legs. Then (Figure 8, time point: 30'), following the same instructions, gas exchange at rest was collected and measured by using a ventilated canopy hood attached to the Quark RMR metabolic cart for 30 minutes. Exactly the same procedures were followed on the second visit.

Steady-state exercise bout

Participants were equipped with a facemask for collecting gas exchange. The gas exchange measurement started 1 min before the beginning of the steady-state test with the participants sitting on the cycle ergometer without pedalling, and connected to the Quark RMR cart. After 1 min of gases recording in resting conditions, the steady-state test at 65% HRR intensity started (Figure 8, time point: 90'; i.e., Pre) and continued (constant intensity) until completing a total of 60 minutes, the moment at which the test finished (Figure 8, time point: 150'; i.e., Post). Gases exchange was continuously measured. Every 5 min, participants were asked to report their fatigue perception using RPE scale.

Resting period after exercise

Participants lay down for 60 minutes where resting gas exchange was measured (using a canopy hood for gases collection) for 30 minutes in two separated 15 minutes stages (Figure 8, time point: 195' to 210' and 225' to 240'; i.e., Post), corresponding to 45 to 60 and 75 to 90 minutes after exercise, respectively.

Gases exchange data

Gas exchange data were downloaded at a sample frequency of 5 seconds, and VO_2 and volume of carbon dioxide production (VCO_2) were used to estimate EE and respiratory exchange ratio ($RER=VCO_2/VO_2$). EE was estimated using Weir's abbreviated equation[28] considering zero nitrogen excretion.

$EE\ (kcal/min) = (1.106 * VCO_2) + (3.941 * VO_2)$

For gas exchange data at rest (i.e., Resting), the first 5 minutes of measurement were discarded[29]. Then, average VO_2, VCO_2, RER and EE Pre and Post exercise were calculated. For gas exchange data during exercise, continuous data (every 10 seconds) from VO_2,

VCO_2, RER, and EE were used to examine potential kinetics point by point differences between morning and evening exercise. Average baseline (i.e., Pre) values were used to adjust gas exchange data during exercise to account for circadian variability in resting gas exchange.

Heart rate

The same heart-rate monitor described in visit 2 was placed in the forearm to measure heart rate during the whole visits 3 and 4.

Skin temperature

A total of 16 thermal iButton™ (iButtons DS 1922 L, Maxim, Dallas, USA) were attached to the skin in different spots upon arrival at visits 3 and 4 (i.e.: forehead, left pectoralis, left elbow region, left index fingertip, left forearm, rear neck central area, right clavicula, right deltoid, right shinbone, right sub-clavicular area, right supra- clavicular area, right thigh, and upper breastbone). Skin temperature measurements were taken every 60s (resolution: 0.0625 ◦C)[30]. The programming of iButtons®, as well as the downloading and initial processing of raw data, were performed using the Temperatus® software[31]. Following the completion of the experiment, the data for each iButton were downloaded every 60 seconds and saved in a CSV file. Anomalies in the data were eliminated by excluding time points where the rate of change from the previous value exceeded the interquartile range between quartiles 1 and 3 (corresponding to percentiles 25 and 75, respectively) for the entire dataset[32]. Subsequently, the data were divided into 5-minute blocks, and the average value calculated, resulting in 12 mean values (one for each 5-minute block during the 60-minute steady-state test). Finally, the overall mean temperature[33], as well as the proximal[34] and distal skin temperatures, were determined using the Temperatus® software[35,36], which is a valid and reliable tool for that purpose[32,37]. The equations used for calculating overall mean temperature, as well as the proximal and distal skin temperatures were[35]:

Overall mean skin temperature = (Forehead*0.07) + (Right Scapula*0.175) + (Left Chest*0.175) + (Right Deltoid*0.07) + (Left Elbow*0.07) + (Left Hand*0.05) + (Right Thigh*0.19) + (Right Gastrocnemius*0.2).

Proximal skin temperature = (Right Thigh*0.383) + (Right Clavicular*0.293) + (Right Abdomen*0.324).

Distal skin temperature = (Left Hand+Right Instep)/2.

Blood samples

Intravenous blood samples from the antecubital vein were taken before (Figure 8, time point: 60'; i.e., Pre), immediately after (Figure 8, time point: 150'; i.e., Post) and 90 minutes after (Figure 8, time point: 240'; i.e., 90 min post) the exercise bout. Two tubes per sample were collected (Tube Vacutainer 5 ml serum+gel PL, SST™, BD Medical). The tubes were left to rest for 45 min at room temperature, then centrifuged at 1300 relative centrifugal force for 10 minutes. The first was stored at 4ºC for 24 – 72 h until analysis. The second was used to prepare serum aliquots and stored at -80ºC until analysis.

Serum parameters

Glucose, triacylglycerol lipase, creatine kinase, lactate dehydrogenase (LDH), triglyceride, total cholesterol, c-reactive protein (CRP), C3 complement and C4 complement were determined in a Beckman Coulter AU5832 analyzer (Beckman Coulter Inc., Brea, CA, USA). The homeostatic model assessment index of insulin resistance (HOMA-IR) was subsequently calculated[38]. Insulin, cortisol, thyrotropin (TSH), free triiodothyronine (T3) and thyroxine (T4) were determined using chemiluminescent immunoassay of Beckman Coulter with a DXI analyzer (Beckman Coulter Inc., Brea, CA, USA).

Control of cofounding variables (visits 3 and 4)

Participants were asked to complete the same pre-experimental conditions described in "control of cofounding variables (visit 2)". For women the phase of their menstrual cycle was registered. Room temperature was controlled to maintain a range between 20-24ºC. Participants followed a standardized diet during the previous day (i.e., Pre 24h) (Figure 7). The ingredients were adequately defined (balanced menu with approximately 55% carbohydrates, 27% fat and 18% proteins), being quantities chosen by the participants ad-libitum. The exercise day, they consumed a standardized meal (i.e.: white bread, cheese, olive oil, and apple or similar fruit) 4 hours before exercise (Figure 8, time point: -240'). The subsequent meal was again standardized and consumed within 1 hour after leaving the laboratory (i.e.: salad with lettuce, tomato, sweet corn, carrot, tuna and olive oil, and potato omelette [egg, potato, and olive oil]) (Figure 8, time point: 300'). They were provided with a template for the register of their food consumption and compliance was checked by the researchers after finishing the study. Similarly, they were instructed to replicate, as closely as possible, the physical activity they engaged in at Post 24h and Post 48h after visit 3, aiming to reproduce it after visit 4. Data from accelerometers were utilized to corroborate this.

Muscular biopsies

Muscular biopsies were taken from the vastus lateralis' distal part of the quadriceps in a sub-sample (6 men and 8 women) before (Figure 8, time point: 60'; i.e., Pre) and immediately after (Figure 8, time point: 150'; i.e., Post) the steady state exercise bout. Biopsies were performed by an experienced surgeon using microbiopsy needles (Achieve Automatic Needle 16G x 15 cm), obtaining ~30 mg per biopsy after previous local anaesthesia with 2% lidocaine. From each time point (Pre and Post), 4 skeletal muscle samples were collected. The first one was treated fresh to study mitochondrial respiration and the rest were immersed in liquid nitrogen and stored at -80ºC until further analysis (i.e.: transcriptomics, proteomics and mitochondrial supercomplexes).

Mitochondrial oxygen consumption

Fresh skeletal samples were preserved six hours in BIOPS medium (10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, 15 mM phosphocreatine, pH 7)[39]. Skeletal muscle samples (~10 mg) were submerged in 1 mg/mL proteinase K solution for 60 s. Then, muscle was homogenized (1: 10, w/v) in isolation buffer (250 mM sucrose, 2 mM EDTA, 10 mM Tris, 0,5% free fatty acids albumin, pH 7.4) at 800 rpm at 4°C with a glass–teflon homogenizer. The homogenate was centrifuged twice at 1,000 g for 5 min at 4°C, and the supernatant was centrifuged at 23,000 g for 10 min at 4°C. Then, the mitochondrial pellet was resuspended in 100 µl of isolation buffer, and a 10 µl aliquot was used for protein determination. The remaining sample was washed with 900 µl of isolation buffer and centrifuged at 13,000 g for 3 min at 4°C. The final crude mitochondrial pellet was resuspended in 90 µl MAS 1× medium [70 mM sucrose, 220 mM mannitol, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA and 0.2% (w/v) fatty acid-free BSA, pH 7.2]. Mitochondrial respiration was measured by using an XFe24 Extracellular Flux Analyzer (Seahorse Bioscience)[40]. Mitochondria were first diluted to the needed concentration required for plating in cold 1× MAS (1µg/µL). Next, 50 µl of mitochondrial suspension was delivered to each well (except for background correction wells) while the plate was on ice. The plate was then centrifuged at 2,000 g for 10 min at 4°C. After centrifugation, 450 µl of 1× MAS + substrate (10 mM succinate for complex II or 2 mM malate, 2 mM glutamate and 10 mM pyruvate for complex I) was added to each well. Respiration by the mitochondria was sequentially measured in a coupled state with substrate present (basal respiration or State 2), followed by State 3o (phosphorylating respiration, in the presence of ADP and substrates); State 4 (non-phosphorylating or resting respiration) was measured after the

addition of oligomycin when all ADP was consumed, and then maximal uncoupler-stimulated respiration (State 3u). The respiration control ratio (RCR) was calculated as State 3o/State 4. Injections were as follows: port A, 50 µl of 40 mM ADP (4 mM final); port B, 55 µl of 30 µg/ml oligomycin (3 µg/ml final); port C, 60 µl of 40 µM FCCP (4 µM final); and port D, 65 µl of 40 µM antimycin A (4 µM final). All data were expressed in pmol/min/µg protein[41,42].

Mitochondrial proteomics analysis

Frozen skeletal muscle samples (~10 mg) were homogenised with 5 strokes in a Potter-Elvehjem tissue grinder attached to a rotating drill (~1000 rpm) in 1 mL solution B (1mM EDTA, 220mM mannitol, 20mM HEPES-KOH [pH = 7.6], 70mM sucrose, 0.5mM PMSF) and spun at 1000 x g for 5 min at 4 °C. The supernatant was further spun at 12,000 x g for 10 min at 4 °C, and the ensuing pellet was stored frozen at −80 °C for subsequent proteomics analysis[43]. The pellet was transferred into a 2 ml centrifuge tube, containing two steel beads, 1X Cocktail with lysis buffer L3 (7M urea, 2M thiourea, 0.2% SDS, 20mM tris, pH 8.0-8.5) and 0.2 M EDTA. After 5 minutes in ice, 10 mM DTT was added in each tube. Samples were homogenised by using a grinder (60 Hz, 2 min), centrifugated at 25,000 g for 15 min at 4°C for 15 minutes. The supernatant was collected and incubated in a water bath at 56°C for 1 hour. 55 mM of IAM was added, and the sample was placed in a dark room for 45 minutes. Cold acetone was added to the protein solution at a ratio of 1:5, and the resultant solution was placed in a refrigerator at -20°C for 30 minutes. The sample was then centrifuged at 25,000 g for 15 min at 4°C for 15 minutes. The resultant pellet was air-dry precipitated by adding lysis buffer (without SDS L), homogenate in a use a grinder (60 Hz, 2 min) to promote protein solubilization. Then, the sample was centrifugated at 25,000 g for 15 min at 4°C. The resultant supernatant was identified as protein solution formass spectrometry experiments.

100 µg of each protein sample was centrifuged at 12,000 g for 20 minutes at 20°C. 100µl 0.5M TEAB was added to the resultant pellet, and then centrifuged at 12,000g for 20 minute at 20°C. Trypsin enzyme was added in a protein in a trypsin/protein ratio 1:20, and the sample was incubated for 4 hours at 37°C. After that, sample was centrifugated at 12,000 g for 15 min at 20°C. The peptide solution after enzymatic digestion was collected at the bottom of the tube. 100µL of 0.5M TEAB was added to the ultrafiltration tube, and centrifuged 12,000g for 15 min at 20°C. After protein digestion, each peptide sample was resuspended in a 200-µl volume of buffer A (2% acetonitrile, can, 0.1% formic acid, FA),

centrifuged at 20,000g for 10 minutes, and the resultant supernatant was taken for injection.

Separation was performed by Thermo UltiMate 3000 UHPLC (Thermo Fisher Scientific, San Jose, CA). The sample was first enriched in trap column and desalted, and then entered a tandem self-packed C18 column (75μm internal diameter, 3μm column size, 25cm column length) and separated at a flow rate of 300nL/min through the following effective gradient: 0~5 minutes , 5% mobile phase B (98% ACN, 0.1% FA); 5~90 minutes, mobile phase B linearly increased from 5% to 26%; 90~100 minutes, mobile phase B increased from 26% to 35%; 100~108 minutes, mobile phase B rose from 35% to 80%; 108~113 minutes, 80% mobile phase B; 113~113.5 minutes, mobile phase B decreased to 5%; 113.5~120 minutes, 5% mobile phase B. The nanoliter liquid phase separation end was directly connected to the mass spectrometer. The peptides separated by liquid phase chromatography were ionized by a nanoESI source and then passed to a tandem mass spectrometer Q-Exactive HF X (Thermo Fisher Scientific, San Jose, CA) for DDA (Data Dependent Acquisition) mode detection. The main parameters were set: ion source voltage was set to 1.9kV, MS1 scanning range was 350~1,500m/z; resolution was set to 60,000; MS2 starting m/z was fixed at 100; resolution was 15,000. The ion screening conditions for MS2 fragmentation: charge 2+ to 6+, and the top 30 parent ions with the peak intensity exceeding 10,000. The ion fragmentation mode was HCD, and the fragment ions were detected in Orbitrap. The dynamic exclusion time was set to 30 seconds.

The raw data was identified using the software MaxQuant (version 1.5.3.30). At the spectrum level, filtering was performed with PSM-level FDR < 1%, and at the protein level, further filtering was performed with Protein-level FDR < 1%. WoLF PSORT software was used to filter the results by subcellular localization. Only proteins that the software predicted to be located at mitochondria were further analyzed. Functional analysis was developed by using GO, KOG, KEGG database. Based on the quantitative values obtained from each protein in multiple replicates of the sample, fold-change was obtained in the comparative groups. P <0.05 in the t-test was considered statistic significant in the comparison between groups. MitoCarta 3.0[44] was used to grouped the proteins in mitochondrial pathways[45].

Transcriptome analysis by RNA-Seq

RNA from skeletal muscle was extracted using Trizol (Invitrogen). The RNAs were precipitated, and their quality and quantity assessed using an Agilent Bioanalyzer 2100 and an RNA 6000 chip (Agilent Technologies). Subsequently, cDNA libraries were constructed using the Hieff NGS™ Ultima Dual-mode mRNA Library Prep Kit (Yeasen), and their quality was checked using an Agilent Bioanalyzer 2100 with a DNA 1000 chip (Agilent Technologies). The libraries were then subjected to Paired End 150 sequencing in a DNBSEQ-G400 system (MGI), with a target of 40M reads per sample. The quality of the resulting sequencing reads was evaluated using FastQC. The GCF_000001405.39_GRCh38.p13 reference human genome was obtained from the NCBI database. Filtering was performed using SOAPnuke, developed independently by BGI, with the following criteria: removal of reads containing adaptor pollution; removal of reads with an N content greater than 5%; and removal of low-quality reads, defined as reads with bases having a quality score less than 15, and with the proportion of total bases in the reads greater than 20% considered as low-quality reads.

The filtered "Clean Reads" were savId in FASTQ format. Subsequently, HISAT was utilized to align reads to each genomic locus. Bowtie2 was employed to map the clean reads to the reference gene sequence (transcriptome), and RSEM was used to calculate the gene expression level for each sample.

Evaluation of supercomplexes formation by BNGE

Supercomplexes levels were analyzed in isolated mitochondria from human muscle biopsis by BNGE (I. Wittig, H. P. Braun, H. Schägger, Blue native PAGE. Nat. Protoc. 1, 418–428 (2006). Mitochondrial proteins were solubilized with 10% digitonin (4 g/g;

Sigma-Aldrich D5628) and run on a 3 to 13% gradient Blue native gel. The gradient gel was prepared in 1.5-mm glass plates using a gradient former connected to a peristaltic pump. After electrophoresis, the gels were further processed for Western blotting. After BNGE, proteins were electroblotted onto polyvinylidene difluoride (PVDF) transfer membrane (Immobilon-FL, 0.45 µm; Merck Millipore, IPFL00010) for 1 hour at 100 V (complex IV) (anti-COI, Invitrogen MTCO1 459600), complex III (anti-UQCR2, Proteintech, 14742-1-AP) and VDAC1 (Abcam, ab15895). The secondary antibodies were goat anti-rabbit IgG (H+ L), Alexa Fluor 680 conjugate (A-21076) from Life Technologies and anti-mouse dylight 800 from Rockland (610145002) and acquired with the ODYSSEY Infrared Imaging System (LI-COR) were incubated for 45 min at RT.

3.7. Statistics

Descriptive statistics of the study subjects are shown as mean ± standard deviation.

Continuous data obtained by specific outcomes related to indirect calorimetry (i.e., VO_2, VCO_2, RER and EE every 10 seconds during exercise) were analyzed using Statistical Parametric Mapping one-dimension (SPM1D)[46] package available for Matlab (v.0.4, that allows conducting conventional statistical tests on one-dimension-al data[46]. Previously, smooth data function in Matlab was applied to all curves to smooth noisy data. Smooth data returns a moving average of the force curve using a fixed window length that is determined heuristically. We performed paired t-tests in SPM1D to compare VO_2, VCO_2, RER and EE data curves during morning vs. evening exercise.

Normal mixed model using random intercepts were used to analyse changes in the following outcomes: interstitial glucose, resting energy metabolism (i.e., VO2, VCO2, EE and RER), physical activity and sleep (i.e., moderate to vigorous physical activity, low physical activity, sedentary behaviour, sleep, total sleep time, WASO, times awake) mitochondrial respiration (i.e., RCR_N, RCR_F, State 3_N, State 3_F, State $4o_N$, State $4o_F$), mitochondrial super complexes (i.e., SC/VDAC), temperature and blood parameters. The models included fixed effects for time and condition (two levels; morning vs evening), as well as the unique patient identifier as a random effect separately for both men and women. A paired t-test was used for comparing morning vs. evening sleep parameters data. Time was described as continuous variables. Significance was set at $P<0.05$. These analyses were performed with R version 3.5.2 using packages 'lme4' and 'lmerTest'. GraphPad Prism version 9.4.1. (GraphPad Software, San Diego, CA) was used to plot the figures.

For transcriptomic analyses, we observed the number of genes changing their expression at Pre in the morning, Post in the morning, Pre in the evening and Post in the evening. Changes in expression levels of genes related to glycogen degradation, cytokines and growth factors were calculated as Post – Pre in the morning, and separately in the evening. Changes in expression levels of circadian genes were calculated as Morning – Evening at Pre, and separately at Post. The DESeq2 method was applied to detect differentially expressed genes. The transcripts that filled the inclusion criteria were then subjected to gene classification, using a databank based on hand-curated literature. The list of differentially expressed genes (DEG) was obtained by ANOVA filtering with FDR correcting with a cut-off of adjusted *P*-value < 0.05. The fold changes are represented as log2FC

(base-2 logarithm of the fold change). The general canonical pathways implicated for the significantly changed transcripts were generated by Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA, USA) before being evaluated and *P*-values <0.05 were considered significant. Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA, USA) was also used for generating the gene expression heatmaps[48].

4. Results

Table 6. Descriptive data of the study subjects

	Men (n=18)				Women (n=17)			
	N	**Mean**		**SD**	**N**	**Mean**		**SD**
General characteristics								
Age	18	27.3	±	7.2	17	25.8	±	6.9
Chronotype	17				17			
Definitely morning type	0				0			
Moderately morning type	4				3			
Neither type	9				12			
Moderately evening type	3				1			
Definitely evening type	1				1			
Anthropometry and body composition								
Body mass index (kg/m^2)	18	24.3	±	2.4	17	22.2	±	1.9
Lean body mass (kg)	18	57.6	±	8.7	17	38.5	±	4.0
Lean mass index (kg/m^2)	18	18.2	±	1.6	17	14.0	±	1.3
Fat mass (kg)	18	15.3	±	4.9	17	19.5	±	3.8
Fat mass index (kg/m^2)	18	4.9	±	1.6	17	7.1	±	1.2
Fat mass (%)	18	20.2	±	5.1	17	32.2	±	4.3
Visceral adipose tissue mass (g)	18	273.7	±	148.3	17	204.2	±	96.7
Apendicular lean mass (kg)	18	25.8	±	5.2	17	15.8	±	2.1
Apendicular lean mass index (kg/m^2)	18	8.1	±	1.1	17	5.8	±	0.8
Cardiorespiratory fitness and physical activity habits								
VO2peak (mL/min)	18	3172.7	±	509.3	15	2073.8	±	361.8
VO2peak/lean mass (mL/kg/min)	18	55.6	±	9.5	15	54.1	±	8.4
Minutes of low physical activity per week	18	533.3	±	974.0	16	356.3	±	264.3
Minutes of moderate physical activity per week	17	329.7	±	454.0	15	173.0	±	230.4
Minutes of vigorous physical activity per week	17	204.4	±	192.0	14	128.6	±	113.1
Minutes of sedentary activities per week	16	2366.9	±	1213.3	14	2180.0	±	989.1

4.1. A single bout of moderate-intensity aerobic exercise in the morning and evening demonstrates similar effects on systemic glucose regulation in both men and women.

In order to study the potential influence of diurnal variations on glucose regulation in response to exercise, a group of healthy men and women (Table 6) performed separated bouts of moderate-intensity aerobic exercise, once in the morning (11:30 a.m.) and once in

the evening (6:30 p.m.) while wearing a continuous glucose monitor (CGM) (Figure 7 and Figure 8). Interstitial glucose levels during the 24 hours before exercise day, the exercise day, and two subsequent days to exercise exhibited a similar response in both morning and evening conditions (Figure 9A-F). When examining diurnal (Figure 9G-L) and nocturnal (Figure 9H-R) glucose levels separately, no distinct differences were observed between morning and evening conditions. However, in men the coefficient of variation of glucose during the diurnal phase follows a different pattern when considering both the exercise stimulus (changes in time) and time of day (changes in condition), with a greater increase at 24 h post-exercise in the evening (time * condition P = 0.04) (Figure 9I).

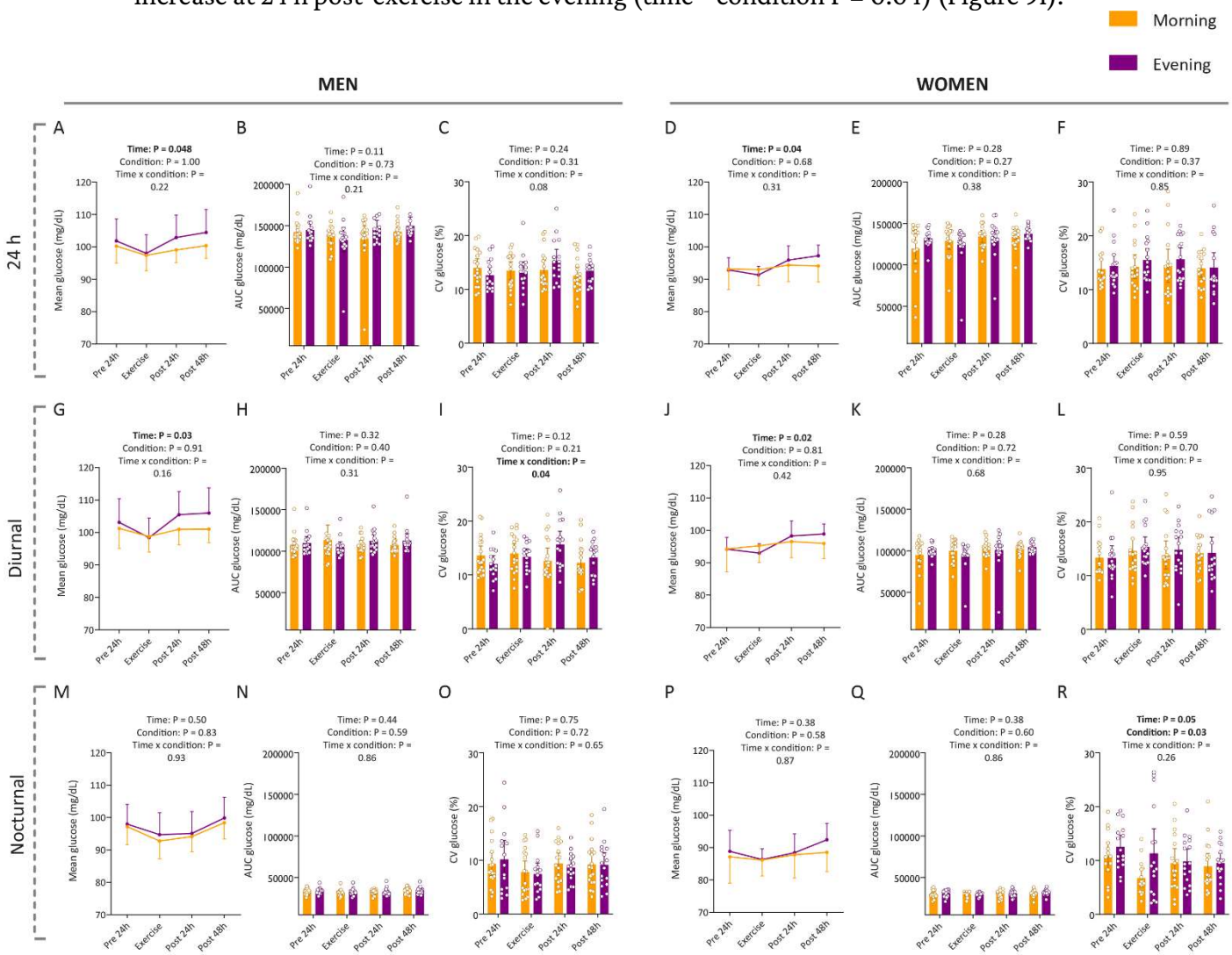


Figure 9. Interstitial glucose levels in response to exercise in the morning and evening conditions. Interstitial glucose levels were assessed during the 24 hours before exercise day (Pre 24h), the exercise day (Exercise), and two subsequent days to exercise (Post 24 h and Post 48 h, respectively). Figures A to F show average data from 24 hours each day. Figures G to L show average data from diurnal glucose (6:00 to 23:59) each day. Figures M to R show average data from nocturnal glucose (00:00 to 5:59) each day. Data from men (Figures A, B, C, G, H, I, M, N, O) and women (Figures D, E, F, J, K, L, P, Q, R) are presented separately. Using normal mixed models to calculate the effect of time, condition and time and condition (time x condition) interaction. Values are mean and CI. CGM: Continuous glucose monitoring; AUC: Area under the curve; CV: coefficient of variation; CI: confident interval.

Other blood parameters (i.e., plasma glucose, insulin and HOMA) were assessed pre- and post-exercise, and 90 minutes post-exercise resulting into no differential effect of exercise and time of day (Table 7 and Table 8). Free living physical activity levels objectively assessed by accelerometery were similar between morning vs. evening exercise conditions the 24 hours preceding exercise, on the exercise day, and in the subsequent days to exercise (Figure 10).

As an overview, the behaviour of glucose in response to exercise is similar in the morning and evening for both men and women. However, in men, there appears to be greater variability in interstitial glucose levels the day after evening exercise.

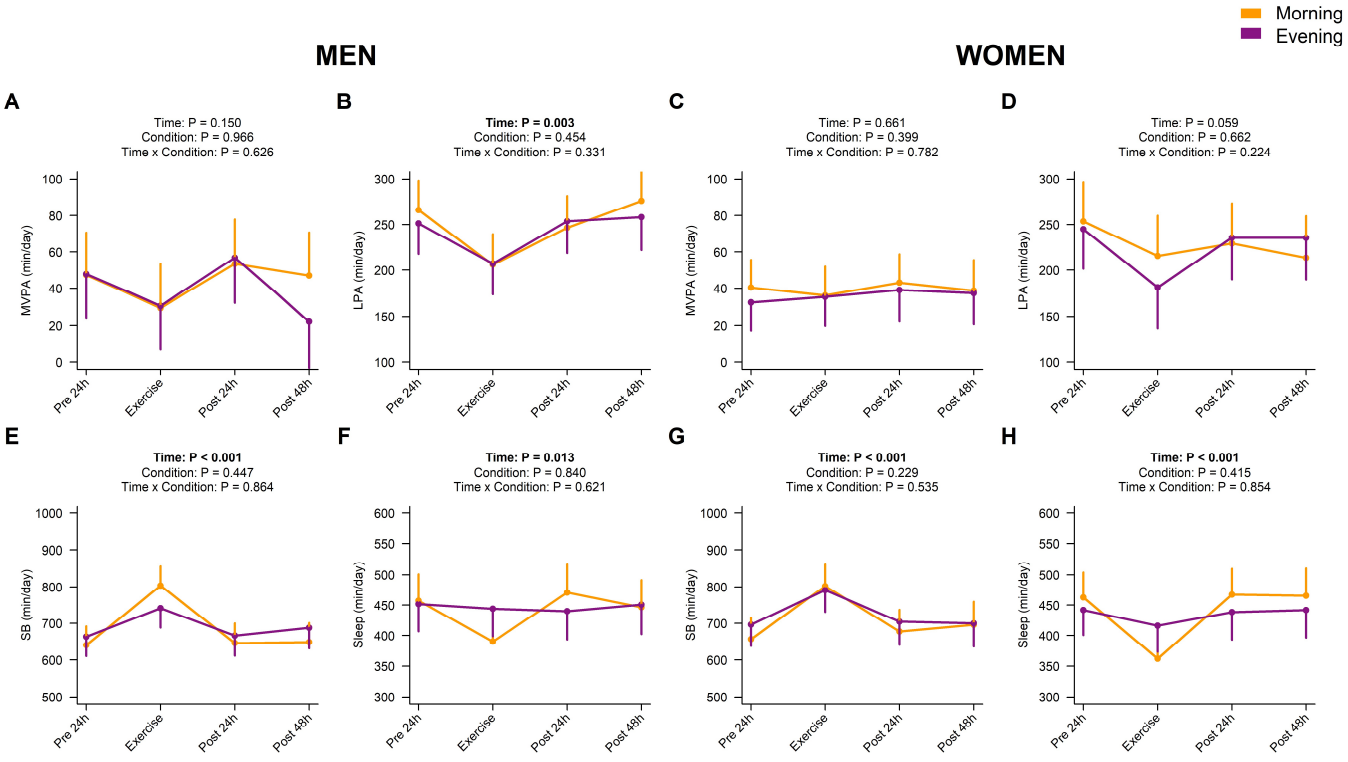


Figure 10. Free living physical activity levels during the 24 hours before exercise day (Pre 24h), the exercise day (Exercise), and two subsequent days to exercise (Post 24 h and Post 48 h, respectively) in the morning and evening conditions. Using normal mixed models to calculate the effect of time, condition and time and condition (time x condition) interaction. Values are mean and CI. Abbreviations: MVPA: Moderate to vigorous physical activity; LPA: Low physical activity; SB: sedentary behaviour.

Table 7. Blood parameters (men)

		Morning			Evening			P-value		
	N	Pre	Post	90 min post	Pre	Post	90 min post	condition	time	time x condition
Glucose (mg/dL)	16	77.1 ± 14.8	78.7 ± 14.8	79.3 ± 14.8	75 ± 14.8	75.7 ± 14.8	77.1 ± 14.8	0.49	0.36	0.99
IBGI ratio	16	6.8 ± 8.6	10.9 ± 8.6	7.5 ± 8.6	7.3 ± 8.6	12.1 ± 9.0	6.1 ± 8.6	0.42	0.45	0.39
HOMA index	18	0.6 ± 1.0	1.0 ± 1.0	0.7 ± 1.0	0.6 ± 1.0	1.1 ± 1.1	0.5 ± 1.0	0.60	0.74	0.50
TGL (U/L)	18	24.5 ± 14.4	26.4 ± 14.4	26.5 ± 14.4	24.5 ± 14.5	26.6 ± 14.6	27.2 ± 14.6	0.87	0.10	0.76
CK (U/L)	18	163.0 ± 207.0	172.0 ± 216.0	164.0 ± 207.0	184.0 ± 215.0	205.0 ± 216.0	197.0 ± 216.0	0.71	0.71	0.81
LDH (U/L)	18	213.0 ± 76.2	242.0 ± 76.2	210.0 ± 78.6	210.0 ± 78.0	231.0 ± 81.0	234.0 ± 81.0	0.22	0.05	0.13
TC (mg/dL)	18	143.0 ± 31.6	157.0 ± 31.6	147.0 ± 31.6	147.0 ± 32.2	158.0 ± 32.4	153.0 ± 32.4	0.83	0.23	0.73
TG (mg/dL)	18	66.5 ± 52.1	77.1 ± 54.5	67.3 ± 54.5	68.7 ± 54.3	91.0 ± 54.5	75.5 ± 54.5	0.84	0.46	0.65
TSH (µUI/mL)	18	1.1 ± 0.8	1.5 ± 0.8	1.3 ± 0.8	1.2 ± 0.8	1.5 ± 0.8	1.3 ± 0.8	0.48	0.73	0.57
T4 (ng/dL)	18	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	0.83	0.69	0.61
T3 (pg/mL)	18	2.8 ± 0.6	2.9 ± 0.6	2.8 ± 0.6	2.8 ± 0.6	2.8 ± 0.6	2.9 ± 0.7	0.31	0.24	0.36
Insulin (µUI/mL)	18	3.2 ± 4.6	5.2 ± 4.6	3.6 ± 4.6	3.2 ± 4.8	5.6 ± 4.8	2.8 ± 4.8	0.52	0.56	0.42
Cortisol (µg/dL)	18	7.2 ± 8.0	10.9 ± 8.0	7.5 ± 8.0	6.2 ± 8.4	7.6 ± 8.7	6.7 ± 8.3	0.32	0.71	0.91
CRP (mg/L)	18	1.4 ± 5.0	1.4 ± 5.0	1.4 ± 5.0	1.7 ± 5.2	1.6 ± 5.2	1.8 ± 5.2	0.85	0.89	0.94
C3C (mg/dL)	18	88.5 ± 19.1	96.9 ± 19.1	89.8 ± 19.6	86.7 ± 19.5	94.9 ± 19.7	91.0 ± 19.7	0.44	0.18	0.52
C4C (mg/dL)	18	18.8 ± 7.2	21.3 ± 7.0	19.1 ± 7.2	18.2 ± 7.2	19.6 ± 7.2	19.5 ± 7.2	0.29	0.23	0.50

Blood samples were obtained pre, post and 90 minutes post exercise in the morning and evening. Data are mean ± SD. Abbreviations: IBGI, Improved basal glucose/insulin; TGL, triacylglycerol lipase; CK, creatine kinase; LDH, lactate dehydrogenase; TC, total cholesterol; TG, triglycerides; TSH, thyroid stimulating hormone; T4, thyroxine; T3, triiodothyronine; CRP, C-reactive protein; C3C, C3 complement; C4C, C4 complement.

Table 8. Blood parameters (women)

		Morning			Evening			P-value		
	N	Pre	Post	90 min post	Pre	Post	90 min post	condition	time	time x condition
Glucose (mg/dL)	16	80 ± 18.9	78.2 ± 18.7	76.8 ± 18.7	76.7 ± 17.8	74.9 ± 17.8	73.2 ± 17.8	0.48	0.21	0.93
IBGI ratio	16	12.0 ± 14.9	12.9 ± 14.8	10.8 ± 15.6	11.1 ± 14.1	13.3 ± 14.1	9.4 ± 14.1	0.98	0.45	0.86
HOMA index	16	1.2 ± 2.1	1.3 ± 2.1	1.0 ± 2.2	1.1 ± 2.0	1.3 ± 2.0	0.7 ± 2.0	0.92	0.25	0.71
TGL (U/L)	16	28.4 ± 18.4	30.6 ± 18.1	29.2 ± 18.3	34.3 ± 18.1	33.8 ± 18.1	31.9 ± 18.1	0.02	0.23	0.27
CK (U/L)	16	157.0 ± 147.0	156.0 ± 142.0	152.0 ± 146.0	129.0 ± 142.0	147.0 ± 142.0	142.0 ± 146.0	0.27	0.49	0.51
LDH (U/L)	16	197.0 ± 95.7	197.0 ± 91.1	189.0 ± 99.9	199.0 ± 91.1	202.0 ± 95.3	202.0 ± 91.1	0.84	0.79	0.57
TC (mg/dL)	16	160.0 ± 43.1	163.0 ± 40.0	164.0 ± 42.7	167.0 ± 40.0	175.0 ± 40.0	169.0 ± 40.0	0.29	0.75	0.82
TG (mg/dL)	16	66.0 ± 58.4	79.9 ± 52.7	75.5 ± 58.1	75.2 ± 52.7	79.9 ± 52.7	62.6 ± 52.7	0.12	0.13	0.07
TSH (µUI/mL)	16	1.3 ± 0.9	1.5 ± 0.8	1.3 ± 0.9	1.1 ± 0.8	1.2 ± 0.8	1.1 ± 0.8	0.39	0.89	0.61
T4 (ng/dL)	16	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.58	0.14	0.28
T3 (pg/mL)	16	2.5 ± 0.4	2.6 ± 0.3	2.5 ± 0.4	2.5 ± 0.3	2.6 ± 0.3	2.5 ± 0.3	0.78	0.63	0.83
Insulin (µUI/mL)	16	6.0 ± 8.9	6.7 ± 8.5	5.2 ± 9.3	5.4 ± 8.5	6.4 ± 8.5	3.9 ± 8.5	0.97	0.23	0.67
Cortisol (µg/dL)	16	8.5 ± 7.4	10.2 ± 7.1	10.4 ± 7.4	7.5 ± 7.1	9.6 ± 7.1	7.0 ± 7.1	0.68	0.69	0.15
CRP (mg/L)	16	0.8 ± 0.9	0.9 ± 0.8	0.8 ± 0.8	0.8 ± 0.8	0.9 ± 0.8	0.8 ± 0.8	0.97	0.88	0.90
C3C (mg/dL)	16	87.0 ± 22.1	90.5 ± 21.5	87.7 ± 22.5	90.4 ± 21.5	93.5 ± 22.0	89.6 ± 21.5	0.38	0.80	0.74
C4C (mg/dL)	16	18.4 ± 6.0	19.6 ± 5.9	19.0 ± 6.0	19.4 ± 5.9	20.1 ± 6.1	19.2 ± 6.0	0.25	0.83	0.45

Blood samples were obtained pre, post and 90 minutes post exercise in the morning and evening. Data are mean ± SD. Abbreviations: IBGI, Improved basal glucose/insulin; TGL, triacylglycerol lipase; CK, creatine kinase; LDH, lactate dehydrogenase; TC, total cholesterol; TG, triglycerides; TSH, thyroid stimulating hormone; T4, thyroxine; T3, triiodothyronine; CRP, C-reactive protein; C3C, C3 complement; C4C, C4 complement

4.2. A single bout of moderate-intensity aerobic exercise in the morning and evening has similar effects on energy metabolism in men and women.

We also investigated EE and substrate oxidation pre-, during and post-exercise in both morning and evening conditions. EE (Figure 11A-D) and substrate oxidation – approached by the respiratory exchange ratio (RER) – (Figure 11E-H) during exercise were similar in the morning and the evening in both men and women. As expected, the increase in EE induced by exercise was maintained at rest at post-exercise, with no different effect of exercise by time of day in men (Figure 11I) and women (Figure 11M). Likewise, resting RER was similar in both men and women (Figure 11J and Figure 11N).

We did not observe a differential effect of exercise by time of day in men in blood lipids or thyroid hormones (Table 7). Results were similar in women, except for blood triglycerides that – although not achieving statistical significance (time * condition P = 0.07) – decreased at 90 min post-exercise more importantly in the evening than in the morning (Table 8).

Skin body temperature exhibited a different pattern between men and women. In men, temperature exhibited a distinct progression from 30 min of exercise onwards, showing a greater increase at this point in the morning compared to evening (Figure 11R) (time * condition P = 0.01). Meanwhile, women started exercising with a higher temperature in the evening, which decreased during exercise, while in the morning it remained stable (Figure 11U) (time * condition P = 0.046).

In the skeletal muscle, we examined mitochondrial respiration through the calculation of the respiratory control ratio by either complex I (RCR_N) (Figure 11K and Figure 11O) and complex II (RCR_F) (Figure 11L and Figure 11P), and total mitochondrial supercomplexes (SC/VDCA) through densitometric analysis (Figure 11Q, Figure 11T and Figure 12). We found no relevant changes in men nor in women when considering the exercise stimulus and time of day. In women, a slightly different response (showing a trend towards significance) was observed in RCR_F that was decreased post-exercise in the evening and increased in the morning (time * condition P = 0.07) (Figure 11Q).

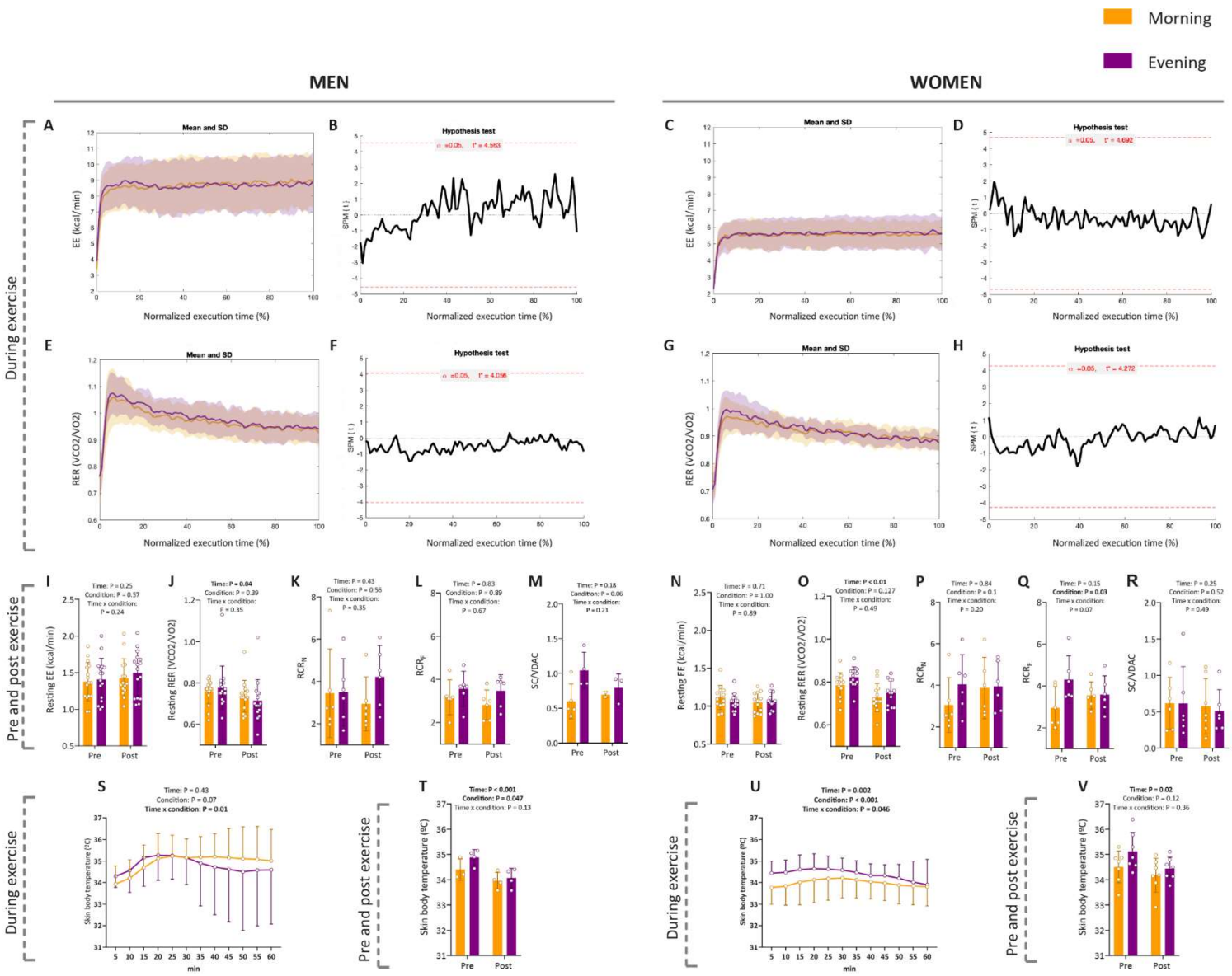


Figure 11. Energy metabolism pre, during and post-exercise in the morning and evening conditions. EE and RER (calculated from VO_2 and VCO_2 exchange) were assessed during exercise and pre and post-exercise at rest (Resting). Figures A, C, and E, G, respectively show SPM1D-analysis for EE and RER during exercise. Solid lines represent mean and shaded areas SD. Figures B, D, F, and H are SPM{t-value} from each corresponding figure (A, C, E, and G). Figures I, M and J, N show EE and RER at rest, respectively. Mitochondrial function was assessed pre and post-exercise. Figures K and O show mitochondrial RCR from complex I (generation of NAD^+). Figures L and P show mitochondrial RCR from complex II (generation of FAD). Figures Q and T show densitometric analysis of the total supercomplexes amount normalized for the VDCA1 signal. Skin body temperature was assessed pre, during and post-exercise. Figures R and T show average temperature values during exercise. Figures S and V show average temperature values at pre and post-exercise. Data from men (Figures A, B, E, F, I, J, K, Q, R, S) and women (Figures C, D, G, H, M, N, O, P, T, U, V) are presented separately. Using normal mixed models for figures I to V (time, condition and time and condition [time x condition] interaction). Values are mean and CI (figures I to V). EE: Energy expenditure; RER: Respiratory exchange ration; VO_2, oxygen volume; VCO_2, carbon dioxide volume; SD: standard deviation; SPM{t-value}: the trajectory Student's t statistic or, equivalently, the mean difference curve normalized by sample-size normalized variance; RCR: respiratory control ratio. NAD^+: nicotinamide adenine dinucleotide (oxidized form); FAD: flavin adenine dinucleotide (oxidized form). VDCA1: Voltage-dependent anion-selective channel 1; AUC: area under the curve; CI: confident interval.

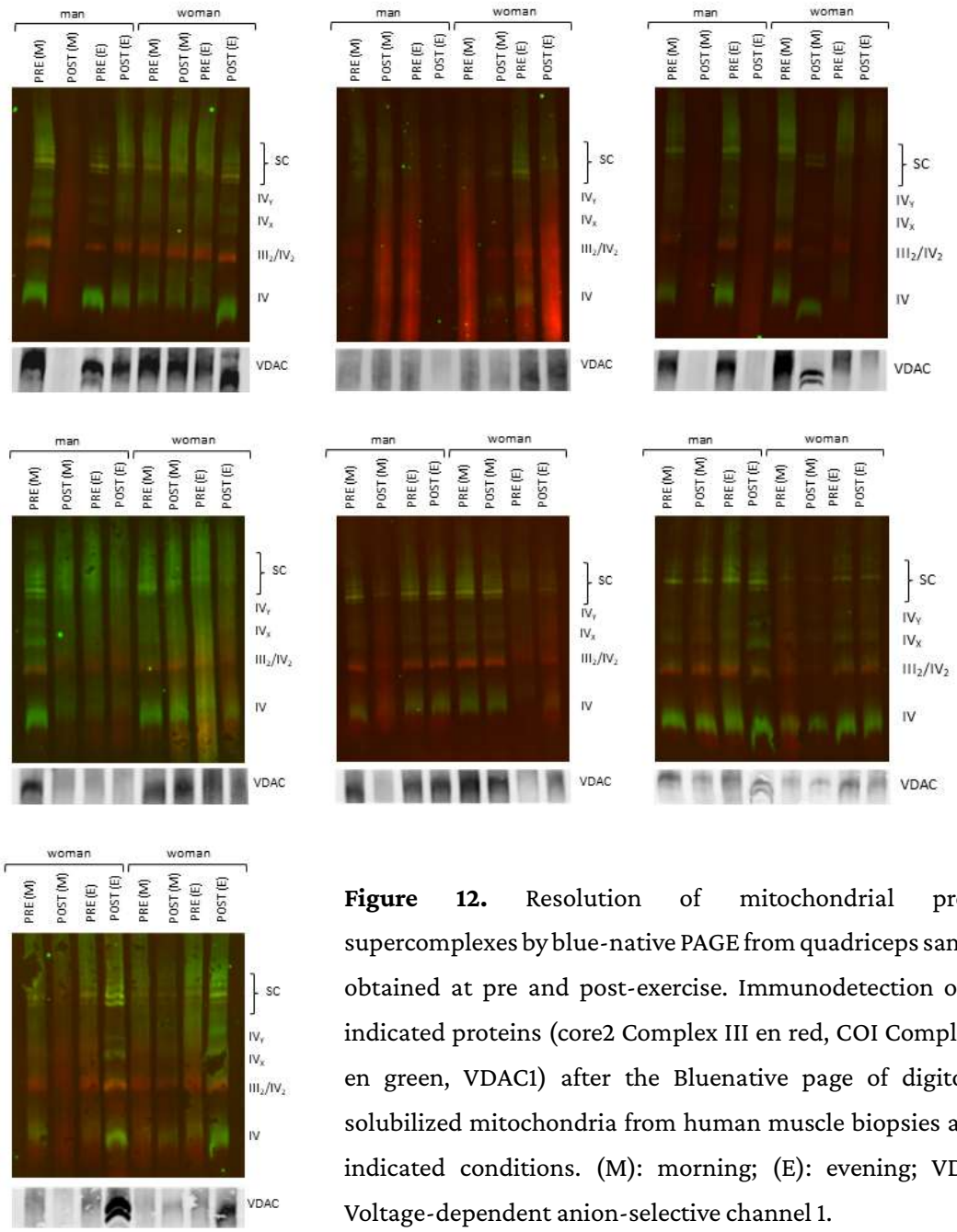


Figure 12. Resolution of mitochondrial protein supercomplexes by blue-native PAGE from quadriceps samples obtained at pre and post-exercise. Immunodetection of the indicated proteins (core2 Complex III en red, COI Complex IV en green, VDAC1) after the Bluenative page of digitonin-solubilized mitochondria from human muscle biopsies at the indicated conditions. (M): morning; (E): evening; VDCA1: Voltage-dependent anion-selective channel 1.

In summary, at systemic level, energy metabolism during and after a single session of moderate aerobic exercise did not display significant variations between morning and evening conditions in both men and women. However, there were contrasting responses between sexes in terms of skin body temperature and mitochondrial respiration in the skeletal muscle.

4.3. A single bout of moderate-intensity aerobic exercise impacts sleep parameters differently according to time of day in women but not men.

We used accelerometers to study the impact of morning vs. evening exercise on sleep parameters on the immediate night (Table 9). In men, total sleep time, wake after sleep onset (WASO), sleep efficiency and times awake were not affected differently by morning or evening exercise, although there was a trend towards significance in total sleep time favouring evening (P = 0.07). In women, we observed a longer total sleep time after evening exercise (P < 0.001) and lees WASO at this time of day, with the latter showing a trend towards significance (P = 0.09); however, no remarkable morning vs. evening variations were noted in sleep efficiency and times awake.

To summarise, exercise had comparable effects on sleep parameters regardless of time of day in men, while women experienced longer sleep after evening exercise.

Table 9. Sleep parameters

	Morning			Evening			
Men	Mean	CI		Mean	CI		P value
Total sleep time (min)	348.7	[306.5 , 391.0]		407.1	[366.6 , 447.5]		0.07
WASO (min)	84.3	[22.0 , 146.6]		77.2	[28.9 , 125.6]		0.80
Sleep efficiency (%)	81.8	[69.0 , 94.6]		80.6	[23.3 , 93.5]		0.29
Times awake (n)	1.8	[0.6 , 3.0]		1.1	[0.4 , 1.7]		0.41
Women							
Total sleep time (min)	389.6	[354.3 , 425.0]		436.1	[382.6 , 489.6]		< 0.001
WASO (min)	69.7	[41.5 , 97.8]		38.6	[27.1 , 50.1]		0.09
Sleep efficiency (%)	84.9	[78.9 , 90.9]		90.4	[87.6 , 93.3]		0.17
Times awake (n)	1.5	[0.3 , 2.7]		1.0	[0.2 , 1.8]		0.86

Abbreviations: WASO, wake after sleep onset; CI, confident Interval. P-value is from a t-test on the paired data for difference.

4.4. Transcriptomic analysis in the skeletal muscle reveals sex and time of day differences in response to a single bout of moderate-intensity aerobic exercise.

To further evaluate the local adaptations in response to acute exercise, we conducted a transcriptomic analysis in the skeletal muscle. Overall, men had less differentially expressed genes than women post-exercise (1,093 vs. 3,343 genes, respectively) (Figure 14A-B). In men, the number of genes modified by exercise only in the morning predominated over those modified only in the evening or commonly in the morning and

evening (Figure 14A). Contrary, in women, the highest number of genes modified by exercise were observed in the evening (Figure 14B). A closer examination of the common exercise-induced modification in the morning and evening revealed different metabolic pathways in men (Figure 14C) and women (Figure 14D). Interestingly, the study of metabolic pathways revealed that genes involved in glycogen and glucose degradation changed their expression only in the evening in both men (Figure 13A and Figure 15C) and women (Figure 13A and Figure 15D). In men, these genes were generally repressed post-exercise at the evening session, while an overexpression was obtained in women (Figure 13A).

In signaling pathways, within the common changes in the morning and evening, we observed a strong activation of pathways linked to inflammation, both in men (Figure 14E) and women (Figure 14F). Interestingly, these were further activated in the evening in women (Figure 15F and Figure 15H) but no relevant time of day variations were observed in men (Figure 15E and Figure 15G). Additionally, changes in cytokines were higher in women than in men at both time of day (Figure 13B). We analyzed the expression of growth factors, which are signaling proteins frequently involved in the regulation of the metabolism and the inflammation process (Figure 13C). Remarkably, GDF15 – a myokine associated to the regulation of mitochondrial metabolism[47] – was upregulated only in women independently of the time of day (Figure 13C). Contrary, there was an overexpression of FGF21 – which is also involved in the control of mitochondrial metabolism[48]– only in men post-exercise in the evening (Figure 13C).

Changes in clock genes after exercise notoriously differed between men and women. CRY2, PER2, RORα and PPARα were repressed only after evening exercise in women; while PER1 was induced by exercise in men, particularly in the evening (Figure 16). When taking into consideration only the morning vs. evening differences without the exercise stimulus (Figures S6), both women and men differentially expressed genes related to the circadian rhythms signaling (Figures S6E-F). Further, independently of the exercise stimulus in both men and women BMAL1 was mainly overexpressed in the evening, while CIART, DBP, HLF, NR1D2, PER1, PER2 and PER3 were mainly repressed in the evening (Figure 13D).

Summarizing these findings together, gene expression in the skeletal muscle revealed sex and time of day differences in metabolic and signaling pathways. These differences can be explained, at least partially, by the different expression of myokines and other growth factors, together with the time-of-day dependent expression of the circadian rhythms' genes.

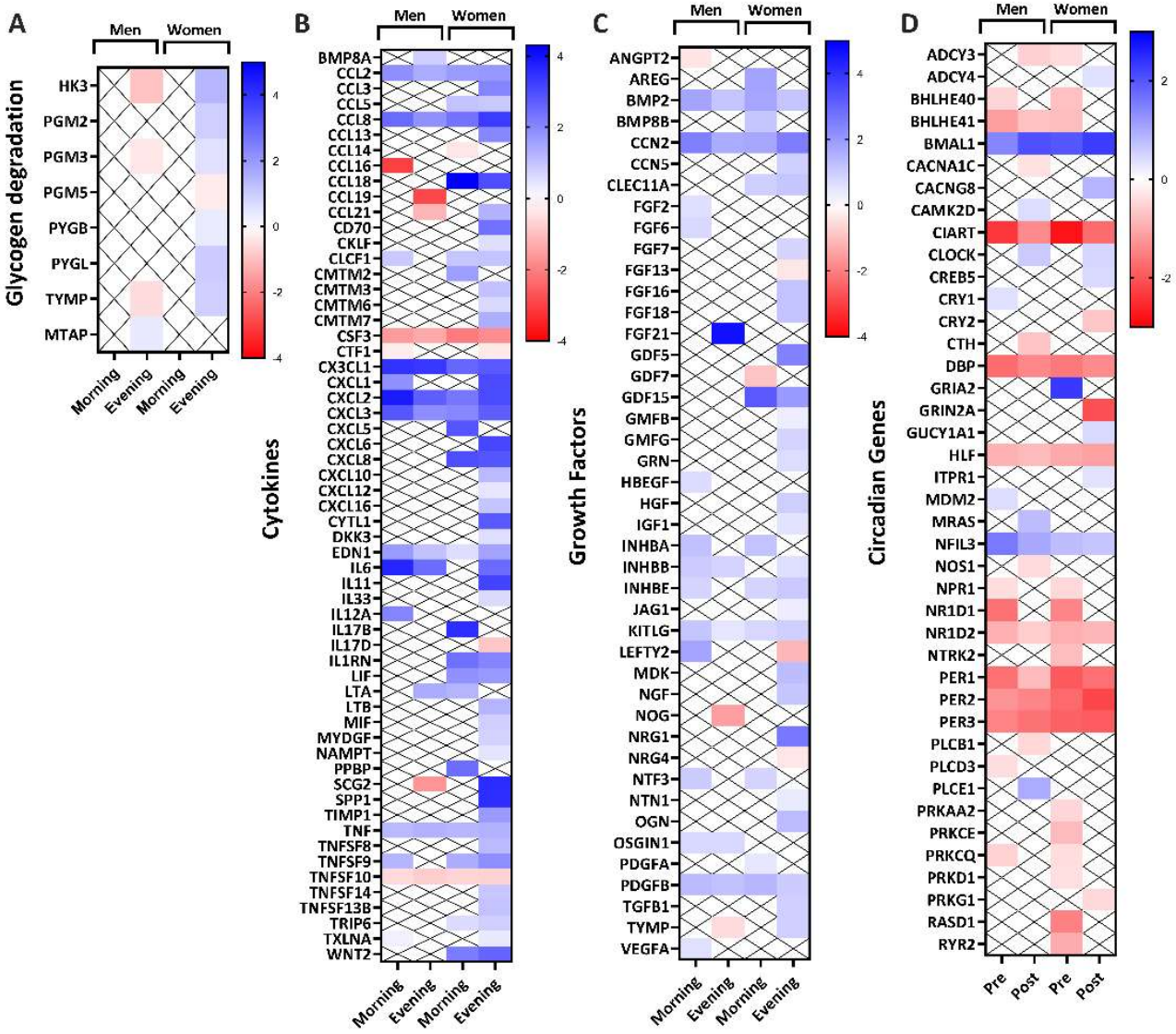


Figure 13. Muscle gene expression profile pre- and post-exercise in the morning and evening conditions. Panels A, B, and C show a representative heatmap of the changes in the expression level from pre to post exercise of the genes related to glycogen degradation (A), cytokines (B) and growth factors (C) in the morning and the evening and in men and women. Panel D show changes in the expression level comparing morning vs. evening of the genes related to circadian rhythm separately pre and post-exercise and in men and women.

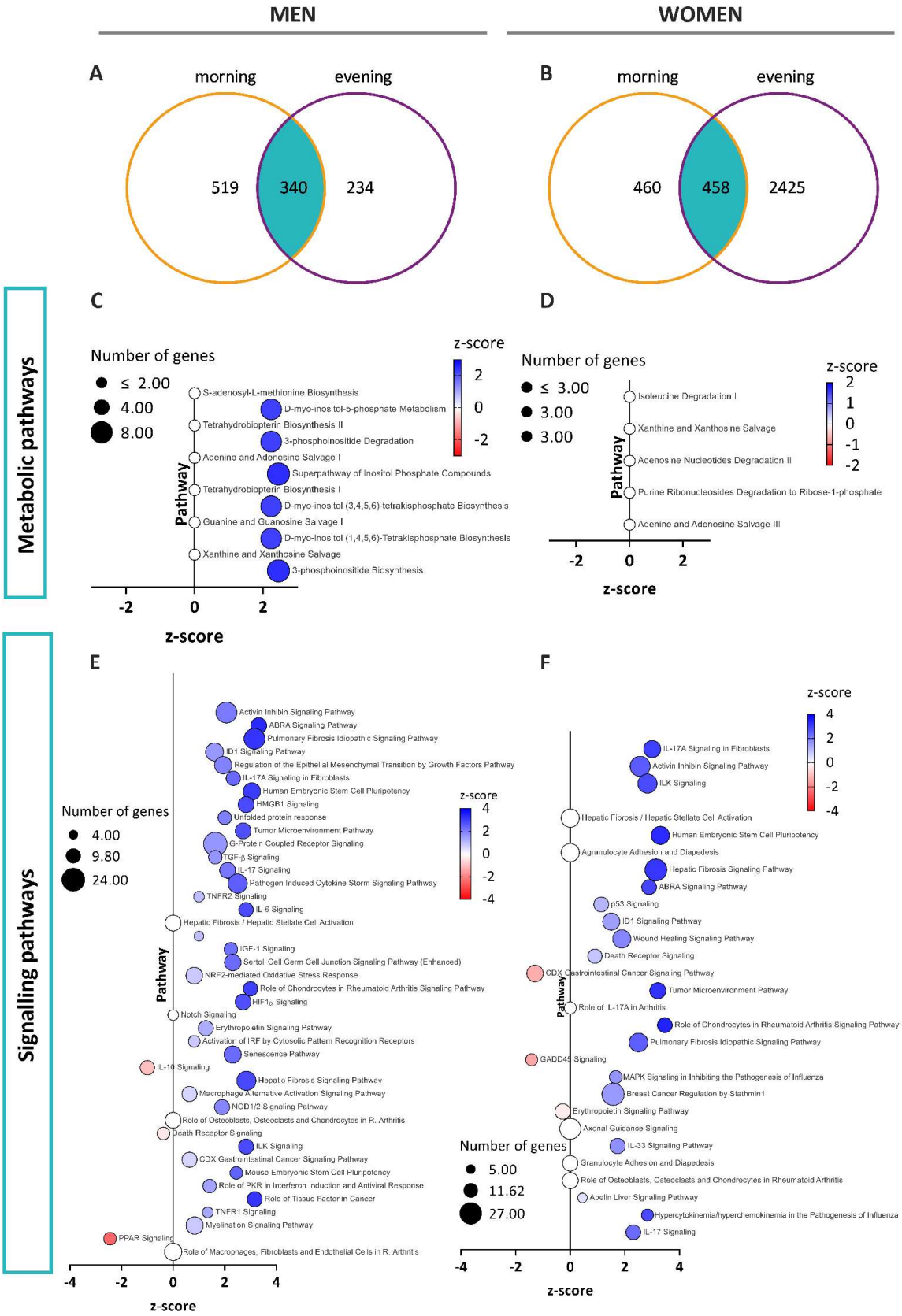


Figure 14. Common gene expression changes post-exercise in morning and evening conditions. Panels A and B show global differences in gene expression after exercise in men (A) and women (B). The orange circle ("morning") represents the number of genes modified by exercise only in the morning. The purple circle ("evening") represents the number of genes modified by exercise only in the evening. The blue segment represents genes modified by exercise commonly in morning and evening conditions. Panels C and D represent the metabolic pathways modified by exercise in both morning and evening conditions in men (C) and women (D). Panels E and F represents of the signalling pathways modified by exercise both in morning and evening conditions in men (E) and women (F).

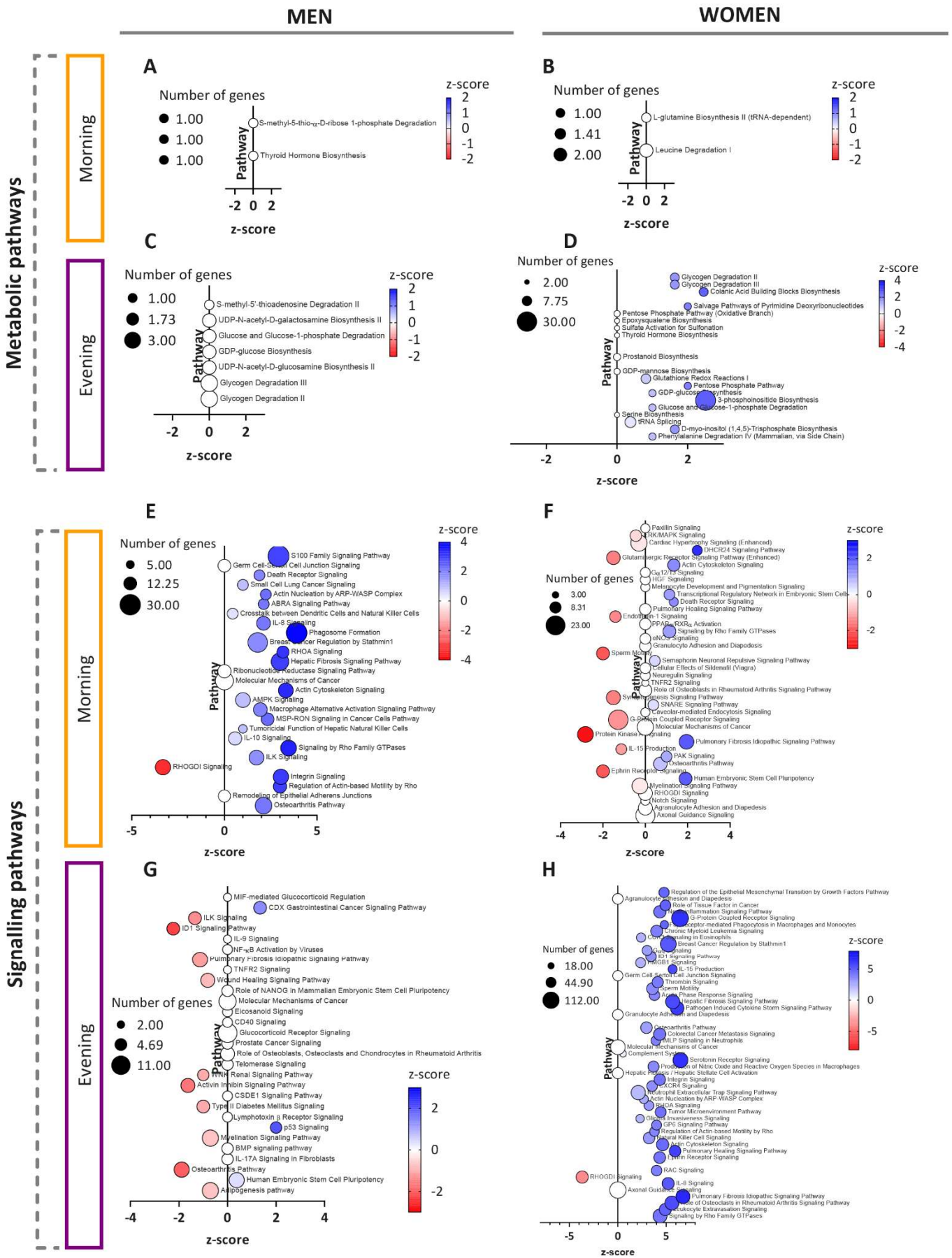


Figure 15. Panels A to D represent the metabolic pathways modified after exercise in men (A, C) and women (B, D) in the morning (A, B) and in the evening (C, D). Panels E to H represent the signalling pathways modified after exercise in men (E, G) and women (F, H) in the morning (E, F) and in the evening (G, H).

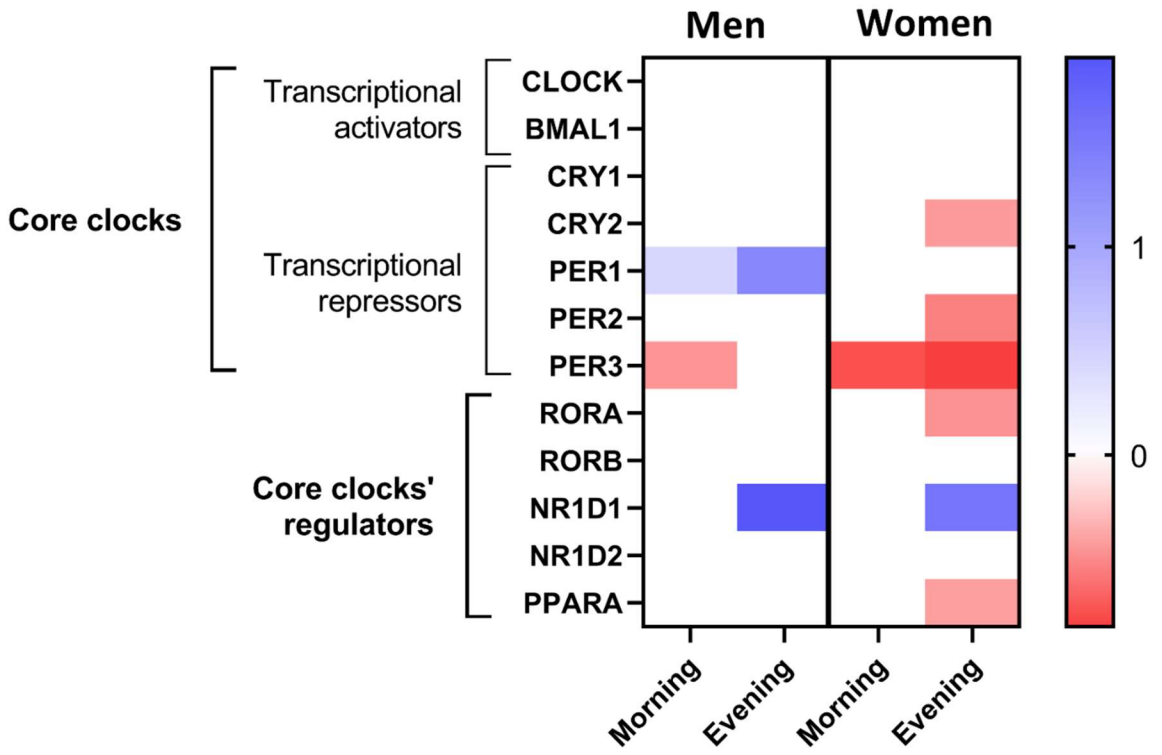


Figure 16. Representative heatmap of changes in clock gene expression pre- and post-exercise in the morning and evening conditions in men and women.

4.5. Sex and time of day differences in response to single bout of moderate-intensity aerobic exercise are observed in the mitochondrial proteome.

In men, the pattern shown in the mitochondrial proteome revealed a general decrease in the mitochondrial proteins post-exercise being more evident in the evening (Figure 17). On the contrary, a general increase in the mitochondrial proteins post-exercise was noted in women, being this increment superior also in the evening (Figure 17).

These changes were reflected in proteins involved in mitochondrial translation (Figure 17A), protein import (Figure 17B), carbohydrate metabolism (Figure 17C), lipid metabolism (Figure 17D), mitochondrial dynamics (Figure 17E), OxPhos system (Figure 17F) and some other mitochondrial functions (Figure 17G). Considering only the time-of-day differences (Figure S8), our analysis revealed diffused pattern regarding the sex differences.

In summary, these results suggest that mitochondrial proteome is also different in response to single bout of moderate-intensity aerobic exercise attending to the sex and the time of day.

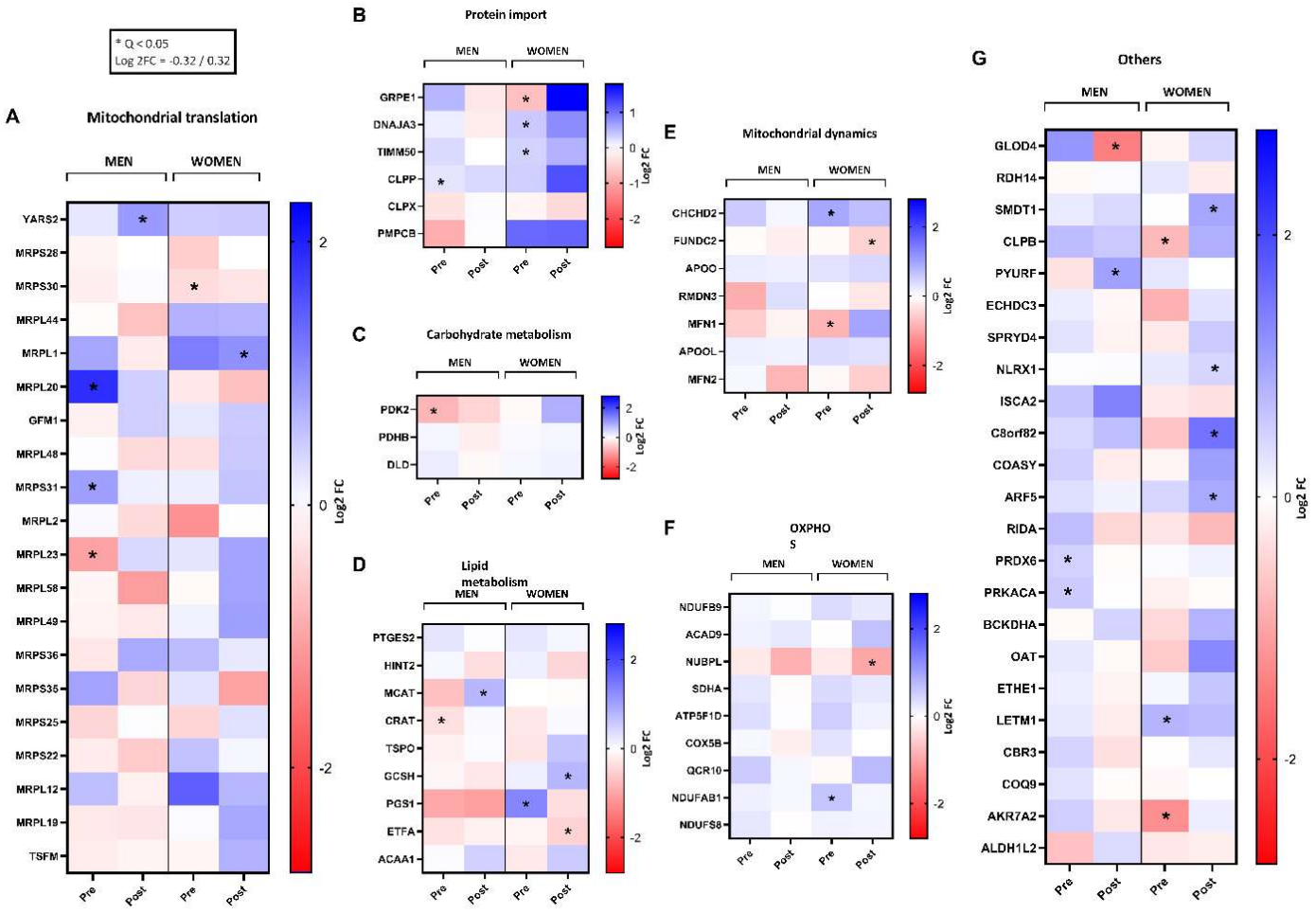


Figure 17. Mitochondrial proteome pre and post-exercise in the morning and evening conditions. Panels A to G show representative heatmaps of the fold change of the protein's levels comparing pre-exercise data versus post-exercise data in the morning (Morning) and pre-exercise data versus post-exercise data in the evening (Evening) in men and women. Proteins are classified according to their functions in mitochondrial translation (A), protein import (B), carbohydrate metabolism (C), lipid metabolism (D), mitochondrial dynamics (E), OXPHOS system (F) and others (G). * P < 0.05. Mitochondrial proteomics was performed in isolated mitochondria from muscle biopsy.

5. Discussion

A single bout of moderate-intensity aerobic exercise in the morning and in the evening overall manifests similar systemic effects on glucose regulation and energy metabolism in both men and women. However, in the skeletal muscle, transcriptional analysis, mitochondrial function, and mitochondrial proteomics revealed a marked sexual dimorphism and time of day variations in response to this bout of exercise. These results emphasize the importance of accounting for sex-specific differences in the

implementation of exercise interventions and the need to conduct separate analyses for both men and women. This study lays the groundwork for a detailed molecular framework, providing a basis for the research on precise exercise prescription in clinical settings.

Considering the influence of molecular clocks in orchestrating glucose and energy metabolism, it is biologically plausible to anticipate distinct responses when exercising in the morning compared to the evening. The present findings – together with other comparable studies in healthy men[49–51] and women[52] – revealed a similar systemic response to a single bout of moderate-intensity aerobic exercise in both interstitial and blood glucose independently of the time of day and sex. Similarly, – in line with analogous studies[53,54] – we observed comparable EE and substrate oxidation response to morning and evening exercise.

Upon detailed examination, our systemic-level findings suggest that men and women could exhibit differential metabolic response to exercise influenced by the time of day. This sexual-dimorphism had already been suggested by previous studies conducted in our laboratory[55–57]. In women, we identified substantial changes (showing proximity to statistical significance) in outcomes such as blood triglyceride levels, which exhibited a greater reduction following evening exercise, and skin body temperature, which was higher in the evening. Higher temperature during exercise has been reported to stimulate endogenous carbohydrate metabolism[58]. Additionally, in women's skeletal muscle, a contrasting pattern in mitochondrial respiration was observed between morning and evening sessions, indicating an increase in respiration through complex II post-exercise in the morning and a decrease in the evening. Last, genes involved in glycogen and glucose degradation were upregulated post-exercise in the evening, accompanied by increased cytokine expression at this time of day. Higher cytokine expression post-evening exercise indicates that, even though the exercise protocol is consistent at both time of day, it may represent a more robust stimulus in the evening[59]. In light of this information, our findings suggest that moderate-intensity aerobic exercise in women induces a more oxidative response in the morning, plausibly with increased fat utilization, while in the evening, a distinct metabolic response with heightened carbohydrate utilization is evident. At first contradictory, mitochondrial proteome in women was specially increased in the evening. Nevertheless, it is reasonable that a negative feedback loop regulation is occurring, wherein translation regulators monitor mitochondrial function and use this information to upregulate or downregulate protein synthesis[60].

Dissimilar results were observed in men. They did not exhibit noteworthy systemic-level variations according to time of day, except for a greater increase in body skin temperature in the morning starting from the minute 30 of the exercise bout. In male skeletal muscle, changes in glycogen and glucose degradation genes were fewer than in women and they were repressed after evening exercise. Neither important time of day differences were found in cytokines. Last, mitochondrial proteome expression was particularly downregulated post-exercise in the evening. Based on this data, it could be hypothesized that evening exercise, more prominently than morning exercise, promotes mitochondrial function while inhibiting carbohydrate utilization. Yet, the comprehensive dataset for men does not evidence clear exercise-induced morning and evening variations. Importantly, diverse response to exercise according to sex could be shaped by the distinct expression of the myokines GDF15 and FGF21 in men and women, which are important regulator myokines of mitochondrial metabolism[47,48].

It is important to emphasize that – when not considering the exercise stimulus, the expression of clock genes (e.g., BMAL1, PER1, PER2, or PER3) consistently changed between morning and evening in both of sexes. This serves as a proof of concept and substantiates the methodological integrity of the study. However, time of day and sex differences revealed by our molecular data were not reflected at the systemic level, a fact that may be explained because (i) we employed a single bout of aerobic exercise (ii) performed at a moderate-intensity, and (iii) in a healthy population. Longer term exercise interventions applying (i) different methodologies (e.g., strength training), (ii) at different intensities (e.g., high-intensity interval training) in (iii) patients with cardiometabolic disturbances (e.g., metabolic syndrome) could potentially reveal whether the molecular alteration noted in skeletal muscle have broader systemic impact. Remarkably, the molecular characteristics we identified in women skeletal muscle align with intervention studies reporting increased fat loss with morning exercise in this population[61,62]. Additionally, one of the studies also reported sex-based differences, with men presenting higher fat oxidation after evening exercise[61]. Higher exercise-intensity would also be expected to translate into more pronounced and observable physiological effects on glucose and energy metabolism as suggested previously[63].

We may expect different results if a similar experiment would be carried out in populations with metabolic diseases[64,65]. Some studies have reported that exercising in the evening potentially prevent hyperglycemic events in patients with type 2 diabetes[66–69]. In contrast, a recent exercise intervention study in middle-aged adults with metabolic

syndrome have shown that morning exercise improves fasting insulin levels to a higher extent than afternoon exercise, yet no between morning vs. afternoon differences were noted in fasting glucose levels[70]. Of note is that these studies analysed the data in men and women together, which hamper to determine the sex-specific exercise-induced effects. The mixed results underscore the importance of a comprehensive exploration into the temporal aspect of exercise, revealing potential differences in outcomes among diverse populations. Future research should explore the impact of metabolic disease and sex differences in the diurnal variation of the physiological and molecular effect of exercise.

6. Conclusion

In conclusion, the present study shows that there are distinct molecular responses to exercise in skeletal muscle based on the time of day and sex, although one single session of moderate-intensity aerobic exercise is not enough to reflect it in systemic adaptations. Our results illustrate that the time of day of exercise impact more importantly on women, resulting exercise a stronger stimulus in the evening and producing a metabolic response with higher reliance on carbohydrates at this time of day. It would be premature to make clinical recommendations in this regard, but here we provide a molecular framework for future research. By identifying and understanding the molecular underpinnings of exercise responses, exercise professionals will be able to make more targeted, personalized prescriptions potentially optimizing therapeutic benefits.

7. References

1. Koronowski KB, Sassone-Corsi P. Communicating clocks shape circadian homeostasis. Science (1979) 2021; 371(6530).

2. Panda S. Circadian physiology of metabolism. Science (1979) 2016:1008–1015.

3. Smith JAB, Murach KA, Dyar KA, et al. nature reviews molecular cell biology Exercise metabolism and adaptation in skeletal muscle. Nat Rev Mol Cell Biol 2023; 24:607–632.

4. Gabriel BM, Zierath JR. Circadian rhythms and exercise — re-setting the clock in metabolic disease. Nat Rev Endocrinol 2019:197–206.

5. Albalak G, Stijntjes M, Wijsman CA, et al. Timing of objectively-collected physical activity in relation to body weight and metabolic health in sedentary older people: a cross-sectional and prospective analysis. Int J Obes (Lond) 2022;46(3):515-522.

6. Van Der Velde JHPM, Boone SC, Winters-Van Eekelen E, et al. Timing of physical activity in relation to liver fat content and insulin resistance H-MRS Proton magnetic resonance spectroscopy ISI Insulin sensitivity index LPA Light physical activity LUMC Leiden University Medical Center MET Metabolic equivalents of t. Diabetologia 2023; 66:461–471.

7. Brito LC, Marin TC, Azevêdo L, et al. Chronobiology of Exercise: Evaluating the Best Time to Exercise for Greater Cardiovascular and Metabolic Benefits. Compr Physiol 2022; 12(3):3621–3639.

8. Sevilla-Lorente R, Carneiro-Barrera A, Molina-Garcia P, et al. Time of the day of exercise impact on cardiovascular disease risk factors in adults: a systematic review and meta-analysis. J Sci Med Sport 2023; 26(3):169–179.

9. R. Ruiz J, Sevilla-Lorente R, Amaro-Gahete FJ. Time for precision exercise prescription: the same timing may not fit all. J Physiol 2023.

10. Amaro-Gahete FJ, Jurado-Fasoli L, Triviño AR, et al. Diurnal variation of maximal fat-oxidation rate in trained male athletes. Int J Sports Physiol Perform 2019; 14(8):1140–1146.

11. Mohebbi H, Azizi M. Maximal fat oxidation at the different exercise intensity in obese and normal weight men in the morning and evening. J Hum Sport Exerc 2011; 6(1):49–58.

12. Sharma P, Agarwal M. Diurnal variation of fat oxidation rate and energy expenditure in an acute bout of endurance exercise by young healthy males. J Family Med Prim Care 2022; 11(1):240.

13. Mode WJA, Slater T, Pinkney MG, et al. Effects of Morning Vs. Evening exercise on appetite, energy intake, performance and metabolism, in lean males and females. Appetite 2023; 182:106422.

14. Ezagouri S, Zwighaft Z, Sobel J, et al. Physiological and Molecular Dissection of Daily Variance in Exercise Capacity Physiological and Molecular Dissection of Daily Variance in Exercise Capacity 2019:78–91.

15. Kim HK, Ando K, Tabata H, et al. Effects of different intensities of endurance exercise in morning and evening on the lipid metabolism response. J Sports Sci Med 2016; 15(3):467–476.

16. Robles-González L, Aguilar-Navarro M, López-Samanes Á, et al. No diurnal variation is present in maximal fat oxidation during exercise in young healthy women: A cross-over study. Eur J Sport Sci 2023; 23(6):936-942.

17. Souissi W, Hammouda O, Ammar A, et al. Higher evening metabolic responses contribute to diurnal variation of self-paced cycling performance. Biol Sport 2022; 39(1):3–9.

18. Cowan SM, Kemp JL, Ardern CL, et al. Sport and exercise medicine/physiotherapy publishing has a gender/sex equity problem: we need action now! Br J Sports Med 2023; 57(7):401–407.

19. Shuhart CR, Yeap SS, Anderson PA, et al. Executive Summary of the 2019 ISCD Position Development Conference on Monitoring Treatment, DXA Cross-calibration and Least Significant Change, Spinal Cord Injury, Peri-prosthetic and Orthopedic Bone Health, Transgender Medicine, and Pediatrics. Journal of Clinical Densitometry 2019; 22(4):453–471.

20. Amaro-Gahete FJ, Sanchez-Delgado G, Jurado-Fasoli L, et al. Assessment of maximal fat oxidation during exercise: A systematic review. Scand J Med Sci Sports 2019; 29(7):910–921.

21. Midgley AW, Mcnaughton LR, Polman R, et al. Criteria for Determination of Maximal Oxygen Uptake A Brief Critique and Recommendations for Future Research. Sports Med 2007; 37(12):1019–1028.

22. Battelino T, Alexander CM, Amiel SA, et al. Continuous glucose monitoring and metrics for clinical trials: an international consensus statement. Lancet Diabetes Endocrinol 2023; 11(1):42–57.

23. Migueles JH, Rowlands A V., Huber F, et al. GGIR: A Research Community–Driven Open Source R Package for Generating Physical Activity and Sleep Outcomes From Multi-Day Raw Accelerometer Data. J Meas Phys Behav 2019; 2(3):188–196.

24. van Hees VT, Renström F, Wright A, et al. Estimation of daily energy expenditure in pregnant and non-pregnant women using a wrist-worn tri-axial accelerometer. PLoS One 2011; 6(7).

25. Hildebrand M, Hansen BH, van Hees VT, et al. Evaluation of raw acceleration sedentary thresholds in children and adults. Scand J Med Sci Sports 2017; 27(12):1814–1823.

26. Hildebrand M, Van Hees VT, Hansen BH, et al. Age group comparability of raw accelerometer output from wrist- and hip-worn monitors. Med Sci Sports Exerc 2014; 46(9):1816–1824.

27. Van Hees VT, Sabia S, Anderson KN, et al. A Novel, Open Access Method to Assess Sleep Duration Using a Wrist-Worn Accelerometer 2015; 10(11):e0142533.

28. Weir JB de V. New methods for calculating metabolic rate with special reference to protein metabolism. J Physiol 1949; (109):1–9.

29. Alcantara JMA, Jurado-Fasoli L, Dote-Montero M, et al. Impact of methods for data selection on the day-to-day reproducibility of resting metabolic rate assessed with four different metabolic carts Nutrition, Metabolism & Cardiovascular Diseases 2023; 33(11):2179-2188.

30. Martinez-Tellez B, Ortiz-Alvarez L, Sanchez-Delgado G, et al. Skin temperature response to a liquid meal intake is different in men than in women. Clin Nutr 2019; 38(3):1339–1347.

31. Martinez-Tellez B, Quesada-Aranda A, Sanchez-Delgado G, et al. Temperatus® software: A new tool to efficiently manage the massive information generated by iButtons. Int J Med Inform 2019; 126:9–18.

32. van Marken Lichtenbelt WD, Daanen HAM, Wouters L, et al. Evaluation of wireless determination of skin temperature using iButtons. Physiol Behav 2006; 88(4–5):489–497.

33. ISO-standard. Ergonomics – evaluation of thermal strain by physiological measurements. Geneva Switzerland Geneva Switzerland: International Standards Organization 2004:1–21.

34. Boon MR, Bakker LEH, Van Der Linden RAD, et al. Supraclavicular skin temperature as a measure of18F-FDG uptake by BAT in human subjects. PLoS One 2014; 9(6):e98822.

35. Martinez-Tellez B, Sanchez-Delgado G, Acosta FM, et al. Differences between the most used equations in BAT-human studies to estimate parameters of skin temperature in young lean men. Nature Scientific Reports 2017; 7(1):10530.

36. Acosta FM, Martinez-Tellez B, Sanchez-Delgado G, et al. Physiological responses to acute cold exposure in young lean men. PLoS One 2018; 13(5):e0196543.

37. Smith ADH, Crabtree DR, Bilzon JLJ, et al. The validity of wireless iButtons® and thermistors for human skin temperature measurement. Physiol Meas 2009; 31(1):95–114.

38. Bonora E, Targher G, Alberiche M, et al. Closely Mirrors the Glucose Clamp. Diabets Care 2000; 23(1):57–63.

39. Fontana-Ayoub, Fasching M, Gnaiger E. Selected media and chemicals for respirometry with mitochondrial preparations. Mitochondrial Physiology Network 2014; 02(17):1–9.

40. Rogers GW, Brand MD, Petrosyan S, et al. High Throughput Microplate Respiratory Measurements Using Minimal Quantities Of Isolated Mitochondria. PLoS One 2011; 6(7):21746.

41. Rogers GW, Brand MD, Petrosyan S, et al. High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. PLoS One 2011; 6(7):e21746.

42. Luna-Sánchez M, Díaz-Casado E, Barca E, et al. The clinical heterogeneity of coenzyme Q 10 deficiency results from genotypic differences in the Coq9 gene. EMBO Mol Med 2015; 7:670–687.

43. Granata C, Caruana NJ, Botella J, et al. High-intensity training induces non-stoichiometric changes in the mitochondrial proteome of human skeletal muscle without reorganisation of respiratory chain content. Nature Communications 2021 12:1 2021; 12(1):1–18.

44. Calvo SE, Clauser KR, Mootha VK. MitoCarta2.0: An updated inventory of mammalian mitochondrial proteins. Nucleic Acids Res 2016; 44(D1):D1251–D1257.

45. González-García P, Díaz-Casado E, Hidalgo-Gutiérrez A, et al. The Q-junction and the inflammatory response are critical pathological and therapeutic factors in CoQ deficiency. Redox Biol 2022; 55:102403.

46. Pataky TC. One-dimensional statistical parametric mapping in Python. Comput Methods Biomech Biomed Engin 2012;15(3):295–301.

47. Chung HK, Ryu D, Kim KS, et al. Growth differentiation factor 15 is a myomitokine governing systemic energy homeostasis. J Cell Biol 2017; 216(1):149–165.

48. Felice V Di, Kumar A, Penna F, et al. FGF21 as Modulator of Metabolism in Health and Disease 2019:10:419.

49. Hobson RM, Clapp EL, Watson P, et al. Exercise capacity in the heat is greater in the morning than in the evening in man. Med Sci Sports Exerc 2009; 41(1):174–180.

50. McIver VJ, Mattin LR, Evans GH, et al. Diurnal influences of fasted and non-fasted brisk walking on gastric emptying rate, metabolic responses, and appetite in healthy males. Appetite 2019; 143:104411.

51. Tanaka Y, Ogata H, Park I, et al. Effect of a single bout of morning or afternoon exercise on glucose fluctuation in young healthy men. Physiol Rep 2021; ;9(7):e14784.

52. Galliven EA, Singh A, Michelson D, et al. Hormonal and metabolic responses to exercise across time of day and menstrual cycle phase. J Appl Physiol 1997; 83(6):1822–1831.

53. Kim HK, Ando K, Tabata H, et al. Effects of Different Intensities of Endurance Exercise in Morning and Evening on the Lipid Metabolism Response. J Sports Sci Med 2016; 15(3):467-476.

54. Mode WJA, Slater T, Pinkney MG, et al. Effects of Morning Vs. Evening exercise on appetite, energy intake, performance and metabolism, in lean males and females. Appetite 2023; 182:106422.

55. Amaro-Gahete FJ, Jurado-Fasoli L, Triviño AR, et al. Diurnal Variation of Maximal Fat-Oxidation Rate in Trained Male Athletes. Int J Sports Physiol Perform 2019; 14(8):1140–1146.

56. Ramírez-Maldonado M, Jurado-Fasoli L, del Coso J, et al. Caffeine increases maximal fat oxidation during a graded exercise test: is there a diurnal variation? J Int Soc Sports Nutr 2021; 18(1):1–9.

57. Robles-González L, Aguilar-Navarro M, López-Samanes Á, et al. No diurnal variation is present in maximal fat oxidation during exercise in young healthy women: A cross-over study. Eur J Sport Sci 2023; 23(6):936–942.

58. Maunder ED, Plews DJ, Merien F, et al. Exercise intensity regulates the effect of heat stress on substrate oxidation rates during exercise. Eur J Sport Sci 2020; 20(7):935–943.

59. Moldoveanu AI, Shephard RJ, Shek PN. The Cytokine Response to Physical Activity and Training. Sports Med 2001 Feb;31(2):115–44.

60. Kummer E, Ban N. Mechanisms and regulation of protein synthesis in mitochondria. Nature Reviews Molecular Cell Biology 2021 22:5 2021; 22(5):307–325.

61. Arciero PJ, Ives SJ, Mohr AE, et al. Morning Exercise Reduces Abdominal Fat and Blood Pressure in Women; Evening Exercise Increases Muscular Performance in Women and Lowers Blood Pressure in Men. Front Physiol 2022; 13:893783.

62. Alizadeh Z, Younespour S, Rajabian Tabesh M, et al. Comparison between the effect of 6 weeks of morning or evening aerobic exercise on appetite and anthropometric indices: a randomized controlled trial. Clin Obes 2017; 7(3):157–165.

63. Gabriel BM, Zierath JR. Circadian rhythms and exercise — re-setting the clock in metabolic disease. Nat Rev Endocrinol 2019:197–206.

64. Loubiere C, Clavel S, Gilleron J, et al. The energy disruptor metformin targets mitochondrial integrity via modification of calcium flux in cancer cells 2017; 7(1):5040.

65. Eshima H, Poole DC, Kano Y. In vivo calcium regulation in diabetic skeletal muscle. Cell Calcium 2014; 56:381–389.

66. Ruegemer, J., Squieres, Ray W., Marsh, Harold M., Haymond, Morey W., Cryer, Philip E., Rizza, Robert A., Miles JM. Differences Between Prebreakfast and Late Afternoon Lgycemic Responses to Exercise in IDDM Patients. J Chem Inf Model 2019; 53(9):1689–1699.

67. Savikj M, Gabriel BM, Alm PS, et al. Afternoon exercise is more efficacious than morning exercise at improving blood glucose levels in individuals with type 2 diabetes: a randomised crossover trial. Diabetologia 2019; 62(2):233–237.

68. Toghi-Eshghi SR, Yardley JE. Morning (Fasting) vs Afternoon Resistance Exercise in Individuals with Type 1 Diabetes: A Randomized Crossover Study. Journal of Clinical Endocrinology and Metabolism 2019; 104(11):5217–5224.

69. Moholdt T, Parr EB, Devlin BL, Debik J, Giskeødegård G, Hawley JA. The effect of morning vs evening exercise training on glycaemic control and serum metabolites in overweight/obese men: a randomised trial. Diabetologia 2021; 64(9):2061-2076.

70. Morales-Palomo F, Moreno-Cabañas A, Alvarez-Jimenez L, et al. Efficacy of morning versus afternoon aerobic exercise training on reducing metabolic syndrome components: A randomized controlled trial Key points. J Physiol 2023:1–15.

Supplementary material for Chapter 4: Sexual dimorphism on the acute effect of exercise in the morning vs. evening: A Randomized Crossover Study

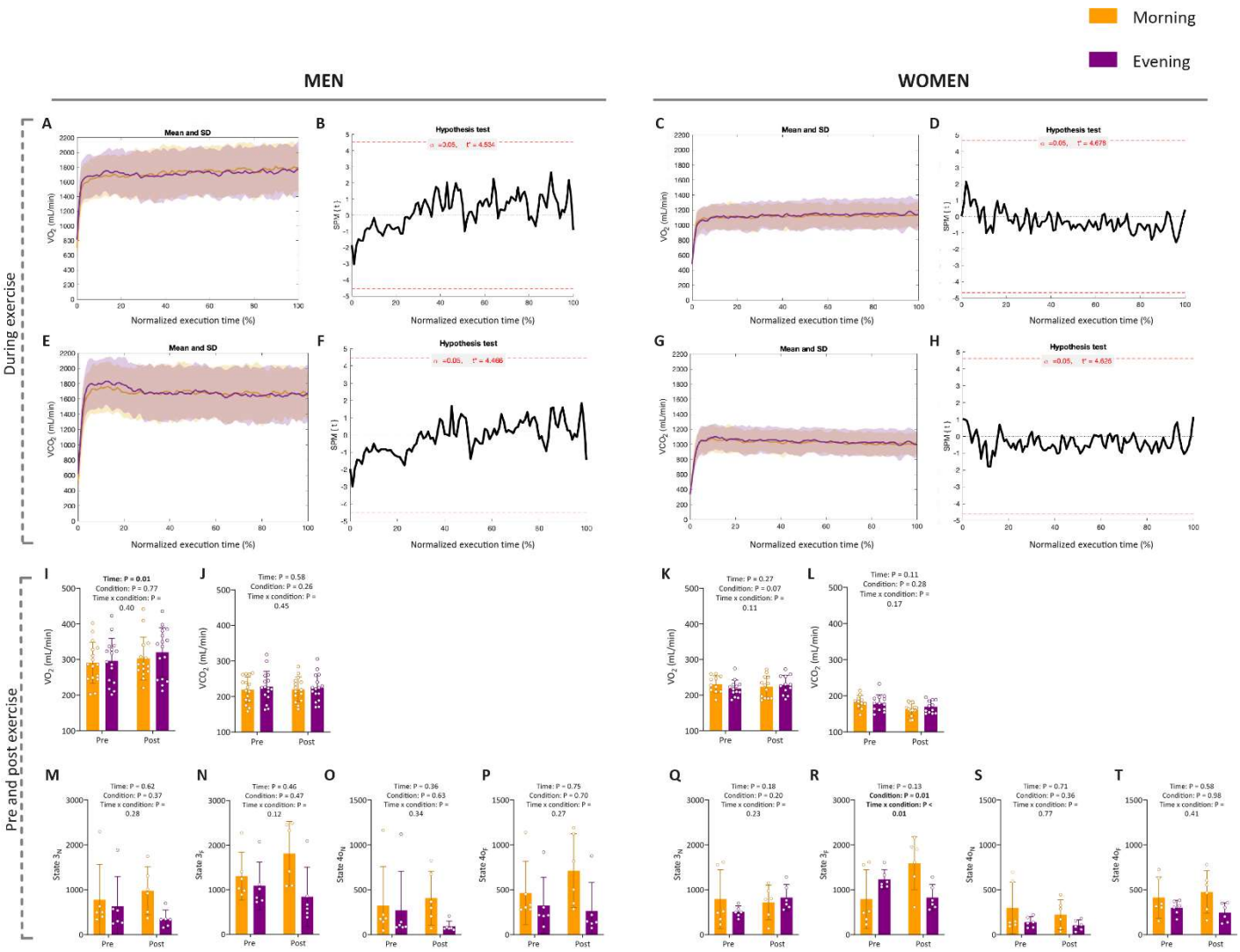


Figure S5. Energy metabolism pre, during and post-exercise in the morning and evening conditions. VO_2 and VCO_2 exchange were assessed during exercise and pre and post-exercise at rest (Resting). Panels A, C, E and G respectively show SPM1D-analysis for VO_2 and VCO_2 during exercise. Solid lines represent mean and shaded areas SD. Panels B, D, F, and H are SPM{t-value} from each corresponding panel (A, C, E, and G). Panels I, K and J, L, show VO_2 and VCO_2 at rest, respectively. Mitochondrial function was assessed pre and post-exercise. Panels M and Q show mitochondrial state 3 from complex I (generation of NAD^+) and panels N and R show mitochondrial state 3 for complex II (generation of FAD). Panels O and S show mitochondrial state 4o from complex I (generation of NAD^+) and panels P and T show mitochondrial state 4o for complex II (generation of FAD). Data from men (A, B, E, F, I, J, M, N, O, P) and women (C, D, E, H, K, L, Q, R, S, T) are presented separately. Using normal mixed models for Panels I to T, time, condition and time and condition (time x condition) interaction. Values are mean and CI (Panels I to T). VO_2, oxygen volume; VCO_2, carbon dioxide volume; SD: standard deviation; SPM{t-value}: the trajectory Student's t statistic or, equivalently, the mean difference curve normalized by sample-size normalized variance; RCR: respiratory control ratio. NAD^+: nicotinamide adenine dinucleotide (oxidized form); FAD: flavin adenine dinucleotide (oxidized form). CI: confident interval.

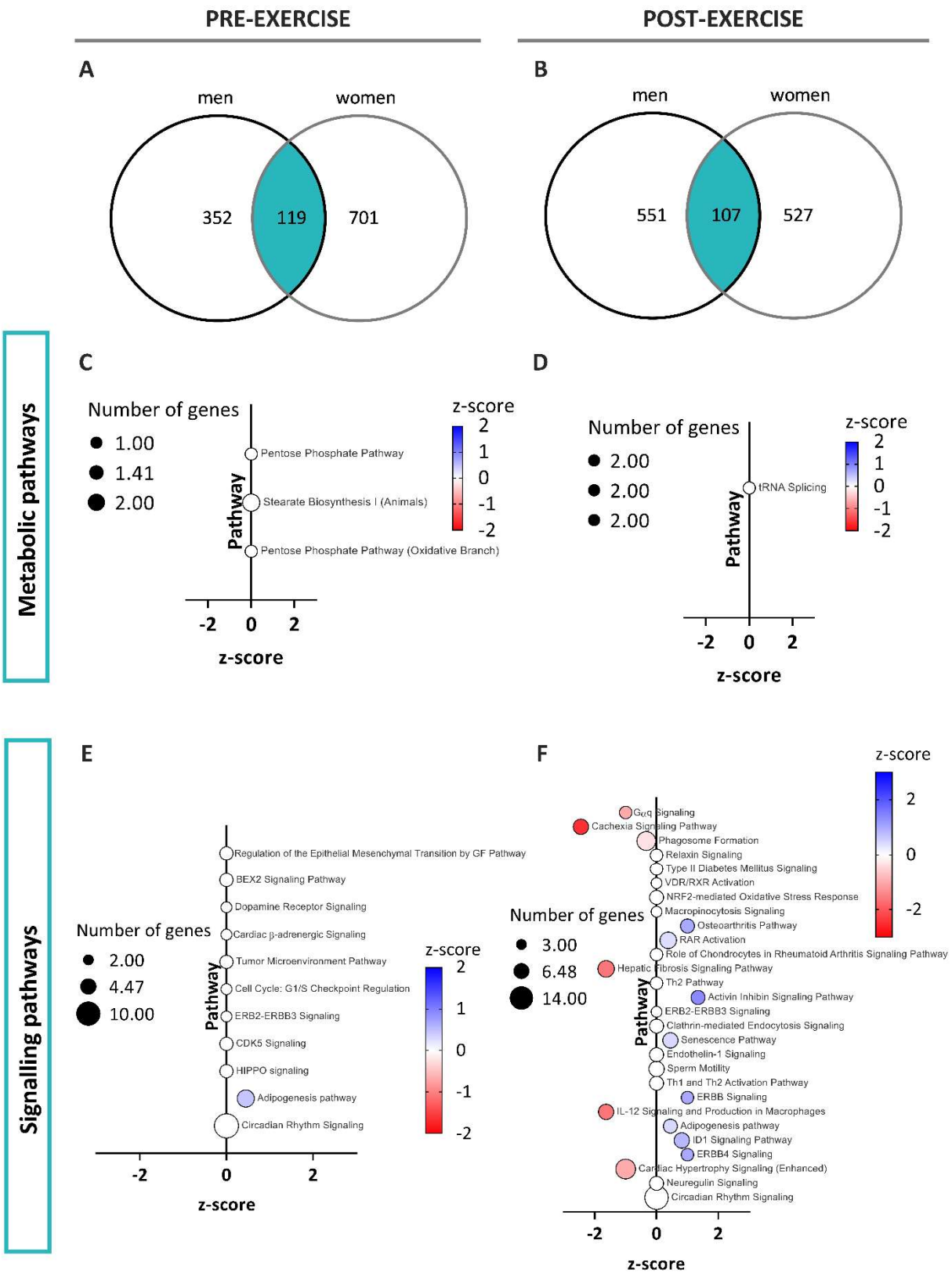


Figure S6. Panels A and B show global differences in gene expression comparing morning vs. evening pre (A) and post-exercise (B). The black circle ("men") represents the number of genes changed after comparing morning vs. evening only in men. The grey circle ("women") represents the number of genes changed after comparing morning vs. evening only in women. The blue segment represents genes changed after comparing morning vs. evening commonly in men and women. Panels C to D represent the metabolic pathways modified in both men and women before (C) and after (D) exercise. Panels E to F represent the signalling pathways modified in both men and women pre (E) and post-exercise (F).

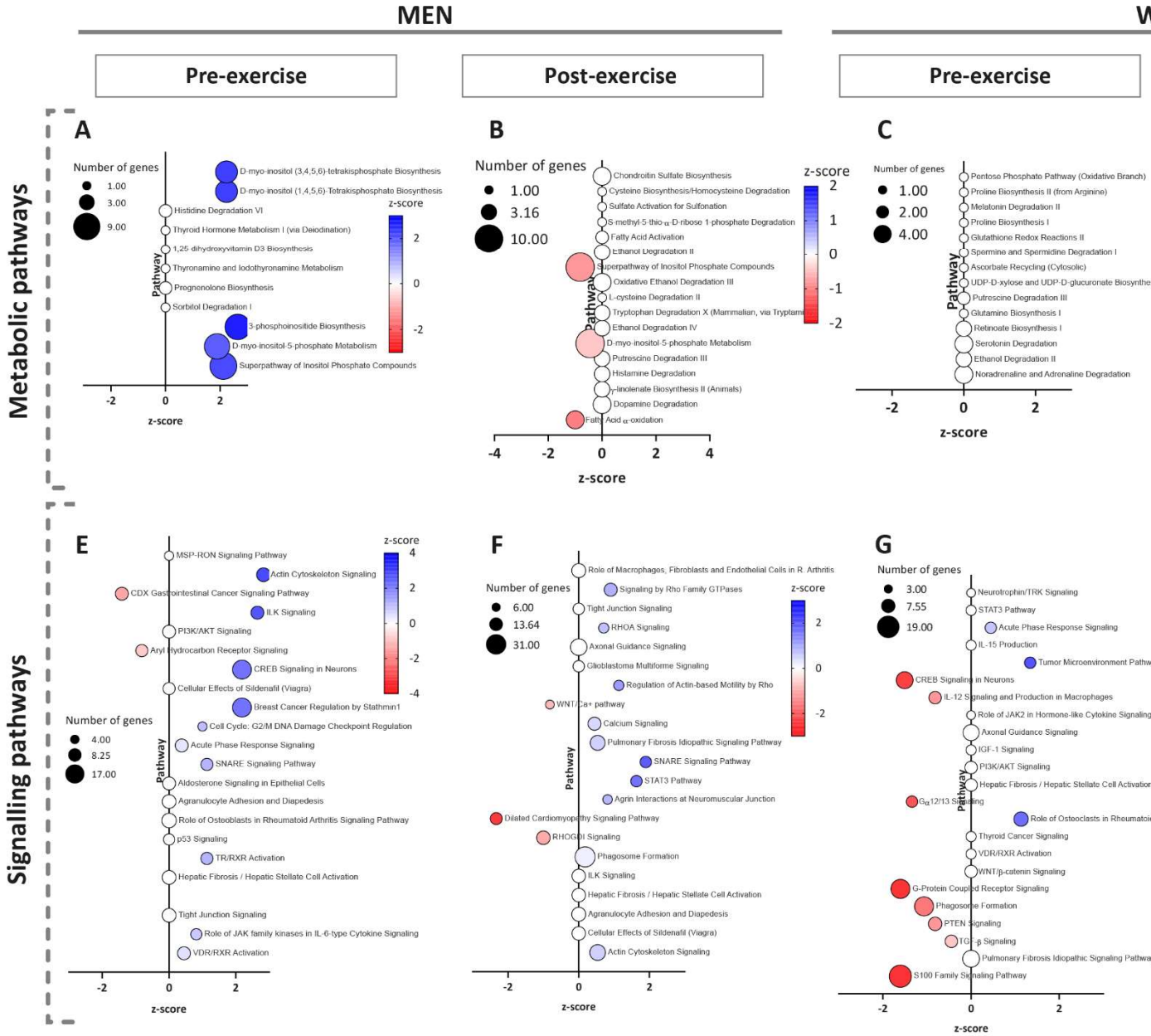


Figure S5. Muscle gene expression profile comparing morning vs. evening conditions. Panels A to D represent the metabolic
D) comparing morning vs. evening conditions in men (A, B) and in women (C, D). Panels E to F represent the signalling pat
comparing morning vs. evening conditions in men (E, F) and in women (G, H).

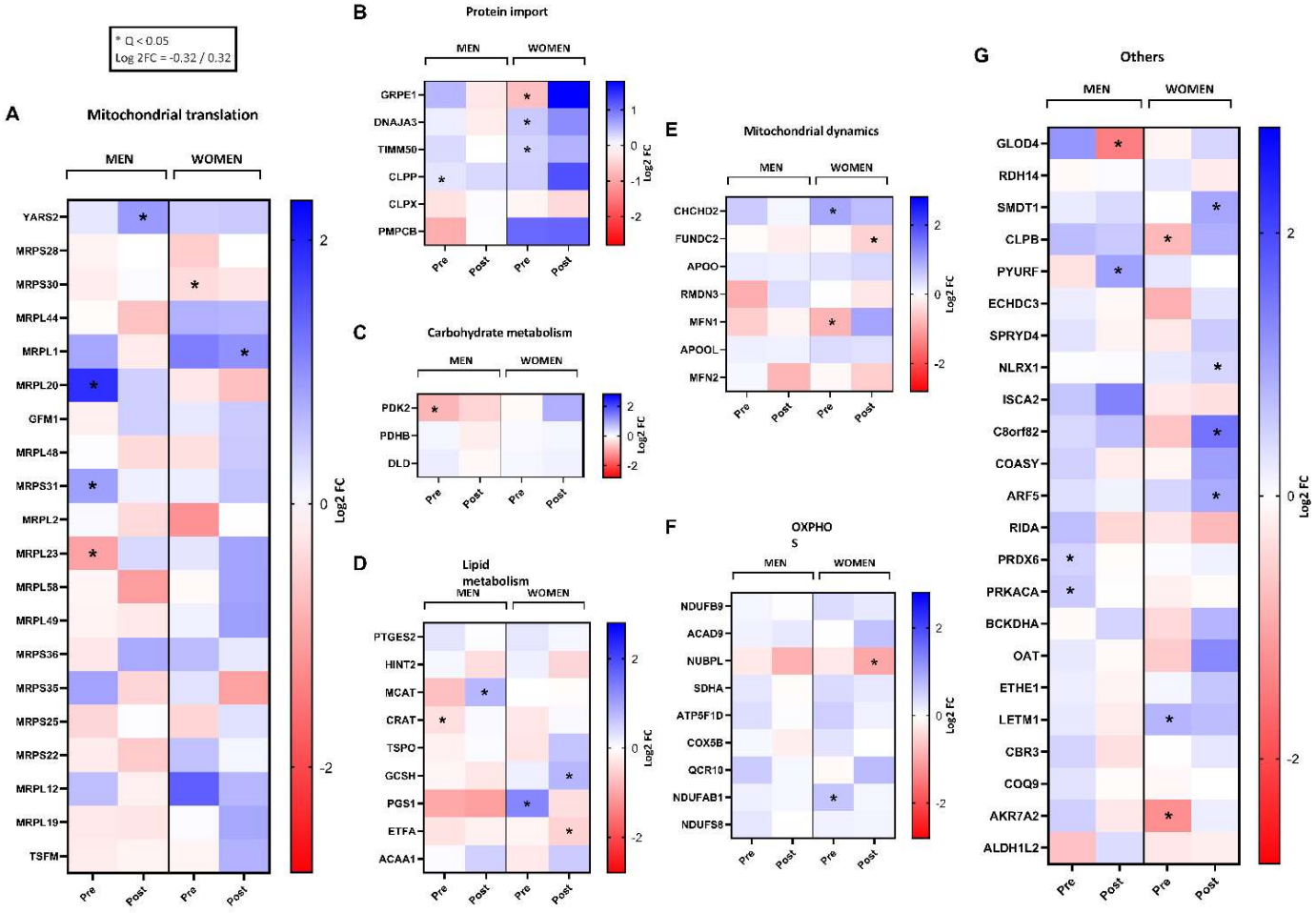


Figure S8. Mitochondrial proteome comparing morning vs. evening conditions pre and post-exercise. Panels E to G show representative heatmaps of the fold change of the protein's levels comparing pre-exercise data in the morning versus pre-exercise data in the evening and post-exercise data in the morning versus post-exercise data in the evening in men and women. Proteins are classified according to their functions in mitochondrial translation (A), protein import (B), carbohydrate metabolism (C), lipid metabolism (D), mitochondrial dynamics (E), OXPHOS system (F) and others (G). *$p <$ 0.05. Mitochondrial proteomics was performed in isolated mitochondria from muscle biopsy.

Chapter 5. Time-of-day impact on the acute effects of moderate intensity aerobic exercise on appetite feelings, Lac-Phe, lactate, and IL-6: role of sex

1. Abstract

Exercise exerts a transient suppressive effect on appetite, attributed to energy-regulatory metabolites such as lactate, interleukin 6 (IL-6), and – according to recent insights – lactate-phenylalanine (Lac-Phe). The degree of appetite suppression following exercise varies considerably among individuals, potentially due to circadian rhythms and biological sex differences. This randomized crossover design study investigated the impact of morning versus evening acute moderate-intensity aerobic exercise on appetite feelings, Lac-Phe, cytosolic non-specific dipeptidase2 (CNDP2), lactate, and IL-6, in 18 men and 17 women. Under standardized conditions, appetite feelings and blood samples were collected pre-exercise, immediately post-exercise, and 90 minutes post-exercise. Skeletal muscle samples were obtained pre- and post-exercise to examine CNDP2 transcriptional expression in a subsample of participants. The response of appetite feelings, Lac-Phe, lactate, and IL-6 to exercise was similar in the morning and evening for both sexes. CNDP2 was induced in the skeletal muscle only after evening exercise in women. Simple linear regressions showed that hunger and fullness were significantly related to Lac-Phe, lactate, and IL-6 in the evening in women, but not in men. In conclusion, evening moderate-intensity aerobic exercise could be more effective than morning for inducing changes on CNDP2 expression and Lac-Phe in healthy women and to modulate their appetite response. In contrast, moderate-intensity aerobic exercise is an insufficient stimulus to impact appetite outcomes in men. These results highlight the relevance of considering sex and time-of-day when designing precise exercise interventions aimed at regulating appetite.

2. Introduction

Obesity is a complex chronic disease in which excess or dysfunctional adiposity impairs health[1]. Likely, the main driver of the rising obesity prevalence in recent decades is the increased food intake and circulating fuels derived from the food environment[2]. In this context, exercise is a highly valuable tool for the prevention and management of obesity, partly due to its role on transiently suppressing appetite[3]. Substantial evidence supports the link between exercise and the regulation of appetite, food intake, and even food choice[4,5]. However, appetite suppression after exercise and exercise-based weight loss

strategies exhibits high inter-individual variability[6,7], making long-term success hard to predict.

Chronobiology is emerging as a principle to consider for developing more precise exercise strategies, given the well-studied and profound connection between molecular clocks (i.e., clock genes) and metabolic health[8]. Indeed, it is possible that some of the variability in weight loss interventions may be explained by the time-of-day that exercise is performed[9–11]. To address this question, it is of interest to investigate how the time-of-day of exercise impact on appetite feelings and to better understand the underlying mechanisms.

Several studies have investigated the effect of the time-of-day of acute exercise on appetite feelings in humans[12–14]. One of these reported increased satiety after morning aerobic moderate-intensity exercise in women with overweight, but not after the same test in the afternoon[14]. Contrary, no morning vs. afternoon/evening differences for appetite feelings or energy intake were found in healthy women after a 60 min aerobic training[13], or in healthy men after 30 min aerobic high-intensity interval training[12]. Mixing results could originate from methodological differences, exercise type/intensity, metabolic status of participants and importantly sex[15]. The time-of-day probably impact the exercise role on energy homeostasis; however, findings are conflicting, and the underlying mechanisms remain unknown.

The suppression of appetite following acute exercise becomes more pronounced with higher exercise intensity[3,16], due to a reduction in orexigenic gut hormones (i.e., acylated ghrelin) and an increase in the anorexigenics (i.e., glucagon-like peptide-1 [GLP-1] and peptite YY [PYY])[3]. Changes in gut hormones have been attributed to the induction of energy-regulatory metabolites, including lactate and interleukin-6 (IL-6)[16,17]. Administration of lactate and IL-6, separately, has demonstrated a reduction in energy intake in humans[18,19]. Lactate promotes the suppression of acylated ghrelin by inhibiting the secretory function of gastric cells[20], while IL-6 contributes by stimulating the secretion of GLP-1 from intestinal L cells and increasing the expression of PYY mRNA[19]. Greater changes in lactate and IL-6 after acute exercise have demonstrated to mediate greater changes in the circulating gut hormones[17]. Additionally, the lactate derived molecule lactate-phenylalanine (Lac-Phe) seems to supress appetite in mice, racehorses and humans[21]. Lac-Phe is synthetised by the ubiquitously expressed cytosolic non-specific dipeptidase2 (CNDP2), and it has emerged as one of the highest upregulated metabolites following exercise[21]. From 2022, the interest on this novel myokine has boosted, positioning it as one potential approach to address obesity[22]. Injections of Lac-Phe (50

mg/kg) reduce food intake by ~50% in diet-induced obese mice and body weight by ~7% after 10 days of treatment[21]. However, the pharmacological doses used in these studies are not comparable to those circulating in blood after an exercise session[22]. Thus, the physiological role of exercise-induced Lac-Phe in appetite regulation in humans is still poorly understood. Only one study addressed this topic in men with obesity, and showed enhanced Lac-Phe levels and decreased hunger after acute moderate continuous exercise, particularly when performed with blood flow restriction[23]. Importantly, levels of Lac-Phe after acute exercise have been associated with a greater reduction in abdominal subcutaneous and visceral fat following 8-week supervised endurance exercise intervention in a mixed group of men and women with obesity[24].

Lac-Phe is therefore a promising metabolite to elucidate the mechanism under the appetite-suppressor effect of exercise and its role on weight management. Moreover, it is a promising tool to predict inter-individual variability in response to exercise-based health interventions. To the best of our knowledge, no study has focused on investigating the effect of the time-of-day on the acute response of Lac-Phe and appetite to exercise separately in both men and women. This study aims to investigate the acute impact of morning vs. evening moderate intensity exercise on perceived appetite feelings, levels of IL-6, lactate and Lac-Phe, and expression of CNDP2 in healthy men and women. By defining the influence sex and time-of-day on exercise metabolic response, it will be possible to develop more precise strategies for obesity prevention and management.

3. Methods

3.1. Subject details

Thirty-five healthy adults (men, n=18; women, n=17) completed the current trial [https://clinicaltrials.gov (NCT05369715)]. Participants mostly presented intermediate and moderate chronotypes. Recruitment was done via advertisements in social networks and at different faculties of the University of Granada (Spain). Inclusion criteria were i) age between 18 and 50 years, ii) body mass index (BMI): 18.5-27 kg/m^2 and iii) practising exercise less than 5 days per week. Exclusion criteria were i) history of disease (i.e., major adverse cardiovascular event, kidney failure, cirrhosis, eating disorder, weight control surgery, HIV/AIDS, rheumatoid arthritis, Parkinson's disease, active cancer treatment in the past year, diabetes mellitus), ii) use of drugs or medications that may affect the results, iii) unstable body weight for 3 months before the start of the study (> 4 kg weight loss or gain), iv) pregnancy and breastfeeding, v) active tobacco abuse or illicit drug use or a history of alcohol abuse treatment, vi) on a special diet or prescribed for other reasons

(e.g., celiac disease). The analyses presented in this study are exploratory in nature and correspond to secondary analyses of a primary study designed to investigate the impact of morning vs. evening moderate-intensity aerobic exercise on glucose regulation and energy metabolism in healthy men and women, and to elucidate molecular mechanisms within skeletal muscle. The sample size for this primary study was calculated to detect differences in fat oxidation during exercise based on results of previous pilot studies conducted in our laboratory. A total of 17 participants are needed to observe statistically significant differences between conditions (morning vs. evening) in fat oxidation during exercise [~10-15%] (80% statistical power and an α of 0.05). In order to conduct the analyses in men and women separately, we finally recruited 18 men and 17 women.

3.2. Study design

The present study is part of the randomised crossover study "DIVA" registered at https://clinicaltrials.gov (NCT05369715). In a randomised and counterbalanced order, each participant performed a single bout of aerobic exercise at two different times of day: (i) morning (i.e., 11:30 a.m.), and (ii) evening (i.e., 6:30 p.m.) with a washout period of at least 3 days in between. This trial strictly followed CONSORT guidelines (http://www.consort-statement.org/consort-statement). All procedures were performed according to the Declaration of Helsinki, and it was approved by the Human Research Ethics Committee of the University of Granada and the Provincial Human Research Ethics Committee (Junta de Andalucía, ref. 1288-N-20). All participants gave their oral and written informed consent. Data for each participant were collected over 4 visits. Figure 7 in Chapter 4 shows the overall design of the study.

3.3. Visit 1. Preliminary examination

Sociodemographic, lifestyle and body composition data were registered, and a medical screening was performed. Participants completed the HÖME questionnaire to determine individual's chronotype (i.e., morningness–eveningness), and the short version of the International Physical Activity Questionnaire (IPAQ) to assess their physical activity levels. Height and weight were measured (no shoes, light clothing) using a model 799 Seca scale and stadiometer (Seca, Hamburg, Germany). Body fat mass and percentage, lean body mass, and visceral adipose tissue (VAT) mass were evaluated by dual X-ray absorptiometry (Discovery Wi, Hologic, Inc, Bedford, MA, USA) and analysed by APEX software. The device was calibrated each day using a lumbar spine phantom. Participants were asked to remain still while being scanned in the supine position, as per guidelines from the International Society of Clinical Densitometry[25]. BMI, lean mass, and fat mass indexes were expressed as kg/m^2.

3.4. Visit 2. Cardiorespiratory fitness

Cardiorespiratory fitness was assessed through a maximal effort test in an Ergoselect 200 cycle ergometer (Ergoline GmbH, Lindenstrasse, Germany). Previous physical activity and diet were controlled (see: "Control of cofounder variables"). A Polar OH1 photoplethysmographic heart-rate monitor (Polar Electro Oy, Kempele, Finland) was placed in the forearm to measure heart rate. After a resting period of 10 minutes, the maximum effort test started with a 3 min stage at 20 watts (W) as a warm-up, followed by increments of 20 W every 3 min until the respiratory exchange ratio (RER) was ≥1 at least for 30s [26]. At this point, further increments of 20 W every 1 min were implemented (with no interruptions) until (i) volitional exhaustion was reached, or (ii) participants had to stop because of peripheral fatigue. Through the maximum effort test, participants' fatigue perception was assessed using a rating of perceived exertion (RPE) scale (Borg CR10 Scale ®). Respiratory gas exchange was measured during the entire exercise test by indirect calorimetry (Quark CPET, Cosmed, Rome, Italy) and collected with a facemask (Hans Rudolph, Inc., Shawnee, KS, USA). According to the manufacturer's recommendations, volume was calibrated using a 3 L calibration syringe and the gas analyzer was calibrated using standard gas concentrations (O_2 = 16%, CO_2 = 5%) immediately before each trial. Resting and maximum heart rate were used to calculate exercise' intensity in the following visits. Gas exchange data were exported from the metabolic cart to an Excel spreadsheet in a sample frequency of 5 sec. Maximal volume of oxygen consumption (VO_2 max) was defined as a respiratory exchange ratio of ≥1.1, once a VO_2 plateau was reached and having attained a heart rate value within 10 beats/min of the individuals' age- predicted maximum (209-0.73 × age)[27] during the maximal effort exercise test. When participants did not achieve the VO_2 max criteria, VO_2 peak was used as the highest VO_2 value that was not an artifact (we screened the data set from the 2nd to the 10th subsequent largest VO2 uptake value). This value was calculated relative to the body mass.

Control of cofounding variables (visit 2)

Participants were asked to complete the following pre-experimental conditions: (i) to refrain vigorous physical activity the previous 48 hours and moderate physical activity the previous 24 hours, (ii) to avoid caffeine ingestion 12 hours before, and (iii) to sleep with normality the night before. In case of women, the phase of their menstrual cycle was registered. Room temperature was controlled to maintain a range between 20-24ºC. Participants attended the laboratory in fasting conditions (4 h) after the ingestion of a complete self-selected meal.

3.5. Visits 3 and 4. Morning and evening moderate-intensity aerobic exercise

On third and fourth visits (Figure 20), participants performed a 60 min steady-state exercise bout on the same cycle ergometer used for the effort test at an intensity of 65% of their heart rate reserve (HRR) either at 11:30 a.m. or 6:30 p.m. The conditions (morning or evening, respectively) were randomized, and visits were separated by a range of 4 to 25 days (median = 7).

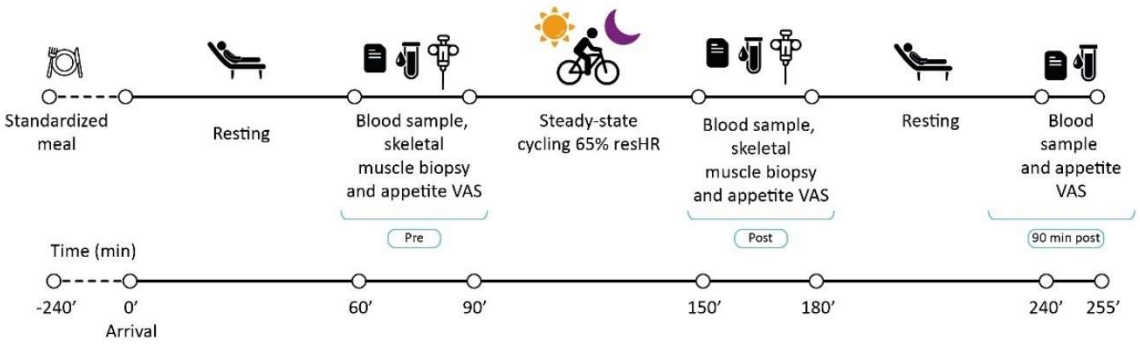


Figure 20. Detailed procedures on visits 3 and 4, where exercise (time point: 90') was performed either at 11:30 or 18:30 in random order. A standardized meal (i.e.: bread, cheese, olive oil and fruit) was consumed 4 hours before arrival (-240'). After arrival (0') there was an acclimatation and resting period until appetite feelings and a first blood sample were collected (60'). Then, participants performed 60 minutes of steady state exercise at 65% resHR (90'). Appetite feelings and a second blood sample after the test (150') were collected. They lay down again to stay 60 minutes resting (180'). To finish, appetite feelings and a third blood sample were completed (240'). A skeletal muscle micro-biopsy was obtained from a subsample of 6 men and 8 women before and

Exercise protocol

After a resting period of 60 minutes (Figure 20, time point: 0'), participants performed a steady-state test at 65% HRR intensity started (Figure 20, time point: 90'; i.e., Pre) and continued (constant intensity) until completing a total of 60 minutes, the moment at which the test finished (Figure 20, time point: 150'; i.e., Post). The same heart-rate monitor described in visit 2 was placed in the forearm to measure heart rate during the whole visits 3 and 4.

Perceived appetite feelings

Perceived appetite feelings were assessed before (Figure 20, time point: 60'; i.e., Pre), immediately after (Figure 20, time point: 150'; i.e., Post) and 90 minutes after (Figure 20, time point: 240'; i.e., 90 min post) the exercise bout. Using a visual analogue scale (VAS) participants were asked to answer the following questions by rating them from 0 to 100 (0 = not at all, 100 = extremely): "How hungry are you?"; "How full do you feel?"; "How

strong is your craving for something tasty?"; "How strong is your craving for something sweet?".

Blood samples

Intravenous blood samples from the antecubital vein were taken before (Figure 20, time point: 60'; i.e., Pre), immediately after (Figure 20, time point: 150'; i.e., Post) and 90 minutes after (Figure 20, time point: 240'; i.e., 90 min post) the exercise bout. Serum (Tube Vacutainer 5 ml serum+gel PL, SST™, BD Medical) and plasma (Tube Vacutainer 5 ml EDTA (100 u)) were collected. The serum tube was left to rest for 45 min at room temperature, then centrifuged at 1300 relative centrifugal force for 10 minutes. The plasma tube was centrifuged immediately at 1300 relative centrifugal force for 10 minutes. Both serum and plasma aliquots were prepared and stored at -80ºC until analysis.

Quantification of IL6

IL-6 levels were determined using the Human Cytokine/Chemokine Magnetic Bead Panel protocol from the Milliplex® Map Kit (Cat. No. HCYTOMAG-60K, Billerica, MA). Briefly, assay plates were washed with wash buffer, sealed, and mixed on an orbital plate shaker for 10 minutes at room temperature. The wash buffer was decanted and the standards, assay buffer, or serum samples were mixed with serum matrix in each well. After the addition of the samples or controls, samples were incubated overnight at 4°C on an orbital shaker with fluorescently-labeled capture antibody-coated beads specific for IL-6. After overnight incubation, well contents were removed via the washing instructions provided by the protocol. Biotinylated detection antibodies were then added to the wells and incubated with samples for 1 hour at room temperature while shaking. After incubation, well contents were removed as previously described and streptavidin-phycoerythrin was added to each well. The samples were incubated with streptavidin-phycoerythrin for 30 minutes at room temperature. After the incubation period, samples were washed as previously described and resuspended in Sheath Fluid. Plates were run on the Luminex MagPix® machine and data were collected using the Luminex xPONENT® software (v. 4.2). Analysis of IL-6 median fluorescent intensity (MFI) was performed using the Milliplex® Analyst software (v. 5.1). The interassay coefficient of variation for IL-6 was 11.93%.

Quantification of lactate and Lac-Phe

The levels of Lac-Phe and Lactate in plasma were analyzed using ultra high-performance liquid chromatography-mass spectrometry (UHPLC-MS)-based metabolomics [28]. Plasma samples (50 µL) were combined with 250 µL of MeOH, vortexed for 30 seconds, and centrifuged for 10 minutes at 16,000 g. The resulting supernatant was vacuum-dried in

200 µL aliquots and then reconstituted in 50 µL of 25% ACN/water solution. Analysis was conducted on a Vanquish UHPLC system coupled to a Q Exactive mass spectrometer (both from Thermo Fisher Scientific, Waltham, MA, USA) operating in negative ion mode, following previously described protocols[28]. Chromatographic separation was carried out using a 2.1 × 100 mm ACQUITYTM UPLC HSS 1.8 µm T3 column (Waters, Milford, MA, USA). The mobile phases consisted of (A) 6.5 mM ammonium bicarbonate in water and (B) 6.5 mM ammonium bicarbonate in 95% MeOH/water. The elution gradient started at 2% B for 1 minute, ramped up linearly to 100% B over 20 minutes, returned to 2% B, and equilibrated for 2.9 minutes (flow rate: 0.35 mL/min, column temperature: 50°C). The Q Exactive mass spectrometer was operated at a resolution of 140,000 in full scan mode with a mass scan range of 70–1050 m/z. Nitrogen sheath gas and auxiliary gas were maintained at flow rates of 45 and 10 arbitrary units, respectively. Capillary and auxiliary gas heater temperatures were set to 300°C and 350°C, respectively, and the spray voltage was 3.00 kV. High-resolution MS/MS spectra of Lac-Phe (m/z = 236.0928) were obtained using parallel reaction monitoring with a resolution of 17,500 and a collision energy of 30 eV. Signal intensities were normalized using the internal standard d5-Phe (615870, Merck, Darmstadt, Germany) at a concentration of 0.8 µg/mL in the extraction solvent.

Control of cofounding variables (visits 3 and 4)

Participants were asked to complete the same pre-experimental conditions described in "control of cofounding variables (visit 2)". For women the phase of their menstrual cycle was registered. Room temperature was controlled to maintain a range between 20-24ºC. Participants followed a standardized diet during the previous day (i.e., Pre 24h) (Figure 20). The ingredients were adequately defined (balanced menu with approximately 55% carbohydrates, 27% fat and 18% proteins), being quantities chosen by the participants ad-libitum. The exercise day, they consumed a standardized meal (i.e.: white bread, cheese, olive oil, and apple or similar fruit) 4 hours before exercise (Figure 20, time point: -240'). The subsequent meal was again standardized and consumed within 1 hour after leaving the laboratory (i.e.: salad with lettuce, tomato, sweet corn, carrot, tuna and olive oil, and potato omelette [egg, potato, and olive oil]) (Figure 20, time point: 300').

Muscular biopsies

Muscular biopsies were taken from the vastus lateralis' distal part of the quadriceps in a sub-sample (6 men and 8 women) before (Figure 20, time point: 60'; i.e., Pre) and immediately after (Figure 20, time point: 150'; i.e., Post) the steady state exercise bout. Biopsies were performed by an experienced surgeon using microbiopsy needles (Achieve Automatic Needle 16G x 15 cm), obtaining ~30 mg per biopsy after previous local

anaesthesia with 2% lidocaine. From each time point (Pre and Post). Samples were immersed in liquid nitrogen and stored at -80ºC until further analysis.

Transcriptome analysis by RNA-Seq

RNA from skeletal muscle was extracted using Trizol (Invitrogen). The RNAs were precipitated, and their quality and quantity assessed using an Agilent Bioanalyzer 2100 and an RNA 6000 chip (Agilent Technologies). Subsequently, cDNA libraries were constructed using the Hieff NGS™ Ultima Dual-mode mRNA Library Prep Kit (Yeasen), and their quality was checked using an Agilent Bioanalyzer 2100 with a DNA 1000 chip (Agilent Technologies). The libraries were then subjected to Paired End 150 sequencing in a DNBSEQ-G400 system (MGI), with a target of 40M reads per sample. The quality of the resulting sequencing reads was evaluated using FastQC. The GCF_000001405.39_GRCh38.p13 reference human genome was obtained from the NCBI database. Filtering was performed using SOAPnuke, developed independently by BGI, with the following criteria: removal of reads containing adaptor pollution; removal of reads with an N content greater than 5%; and removal of low-quality reads, defined as reads with bases having a quality score less than 15, and with the proportion of total bases in the reads greater than 20% considered as low-quality reads.

The filtered "Clean Reads" were savId in FASTQ format. Subsequently, HISAT was utilized to align reads to each genomic locus. Bowtie2 was employed to map the clean reads to the reference gene sequence (transcriptome), and RSEM was used to calculate the gene expression level for each sample.

3.6. Statistical analysis

Descriptive statistics of the study participants are shown as mean ± standard deviation. The normality of the data was assessed using the Q-Q plot method. For variables that did not follow a normal distribution (i.e., IL-6), a log10 transformation was applied. Normal mixed model using random intercepts were used to analyse changes in the perceived appetite feelings, Lac-phe, lactate and IL-6. The models included fixed effects for time and condition (two levels; morning vs evening), as well as the unique patient identifier as a random effect separately for both men and women. Time was described as continuous variable. For post-hoc analyses, time was described as categorical variable. Significance was set at $P<0.05$. These analyses were performed with R version 3.5.2 using packages 'lme4' and 'lmerTest'. GraphPad Prism version 9.4.1. (GraphPad Software, San Diego, CA) was used to plot the figures. For transcriptomic analyses, we observed the number of genes changing their expression at Pre in the morning, Post in the morning, Pre in the evening and Post in the evening. Changes in expression levels of CNDP2 were calculated

as Post – Pre in the morning, and separately in the evening. The DESeq2 method was applied to detect differentially expressed genes. The transcripts that filled the inclusion criteria were then subjected to gene classification, using a databank based on hand-curated literature. The list of differentially expressed genes (DEG) was obtained by ANOVA filtering with FDR correcting with a cut-off of adjusted *P*-value < 0.05. The fold changes are represented as log2FC (base-2 logarithm of the fold change). Moreover, pearson correlation analysis was conducted to examine the relationship between post and 90 min post values in appetite feelings, Lac-Phe, lactate and IL-6 levels in response to exercise in the morning and the evening.

4. Results

4.1. Perceived appetite feelings after a single bout of moderate-intensity aerobic exercise are not dependent on the time-of-day in men and women

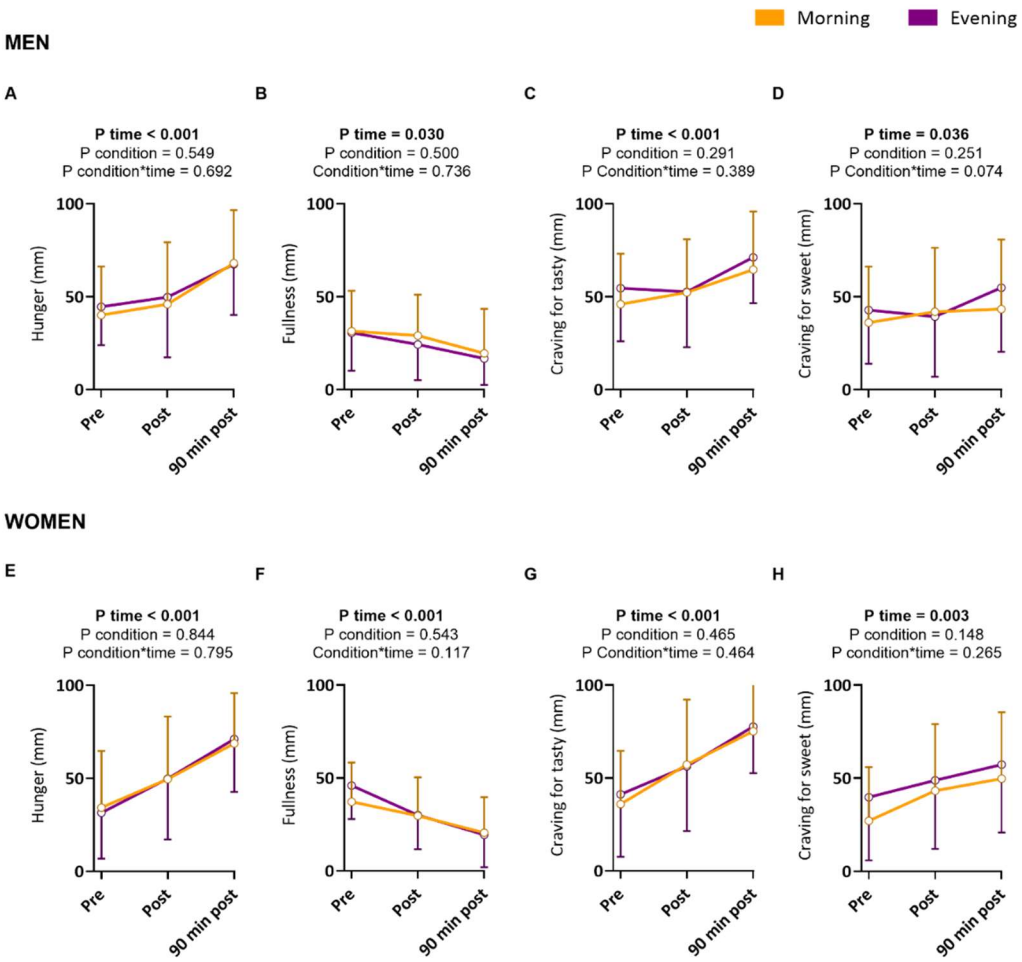


Figure 21. Perceived appetite feelings pre, post and 90 min post-exercise in the morning and evening conditions in men and women. Data from men (Figures A - D) and women (Figures E - H) are presented separately. Using normal mixed models considering time a continuous variable (time, condition and time and condition [time x condition] interaction). Values are mean and CI.

A total of 18 men and 17 women (Chapter 4, Table 6) completed separated bouts of moderate-intensity aerobic exercise, once in the morning (11:30 a.m.) and once in the evening (6:30 p.m.). Perceived appetite feelings (i.e., hunger, fullness, craving for tasty and craving for sweet) were measured pre, post and 90 min post-exercise.

Hunger, craving for tasty and craving for sweet increased significantly after exercise, while fullness decreased (all P time < 0.05), but these responses did not differ significantly between morning and evening conditions (all P condition x time > 0.05) (Figure 21). Only in men, craving for sweet was higher 90 min post evening exercise (Figure 21D), and this was close to reach statistical significance (P = condition x time 0.07).

4.2. Lac-Phe and CNDP2 expression after a moderate-intensity aerobic exercise display time-of-day differences particularly in women

Blood metabolites were analysed at pre, post and 90 min post-exercise (Figure 22). Lac-Phe was not affected by exercise in men, independently of the time-of-day (P time = 0.934, P time x condition = 0.452) (Figure 22A). In contrast, Lac-Phe response to exercise in women displayed a time-of-day dependent pattern (Figure 22E). Baseline levels of Lac-Phe were higher in the evening than morning (P condition = 0.007). Both in the morning and evening, Lac-Phe increased immediately after exercise and decreased at 90 min post (P time = 0.003). The response of Lac-Phe to exercise differed between morning and evening, approaching statistical significance (P condition x time = 0.052). Post-hoc analyses revealed that at 90 min post exercise in the morning Lac-Phe levels returned to baseline values [$pre_{morning}$ = 1.01 µg/L (CI = 0.78, 1.24); 90 min $post_{morning}$ = 0.97 µg/L (CI = 0.76, 1.16)], while in the evening condition Lac-Phe was decreased below the baseline [$pre_{evening}$ = 1.47 µg/L (CI = 1.17, 1.76); 90 min $post_{evening}$ = 0.91 ug/L (CI = 0.73, 1.08)] (P condition x time = 0.019). In the skeletal muscle, we examined the expression of the gene CNDP2 at pre and post exercise (Figure 22B and Figure 22F). We observed a significant overexpression after evening exercise in women (P = 0.047) (Figure 22F) but no significant overexpression in men either after the morning or the evening session (Figure 22B).

In summary, levels of Lac-Phe were affected by moderate-intensity aerobic exercise only in women. Moreover, the female's response to exercise showed a time-of-day dependent pattern in both plasma levels of Lac-Phe and the expression of CNDP2, which was increased only in the evening.

4.3. No time-of-day differences on the acute effect of moderate-intensity exercise on lactate in men and women.

We observed in men an immediate increase in lactate post-exercise and subsequent decrease at 90 min post (P time = 0.023) (Figure 22C), but not in women (P time = 0.801) (Figure 22G). However, there were no time-of-day differences on the acute response of lactate to exercise protocol (all P time x condition > 0.2) in both men (Figure 22C) and women (Figure 22G).

4.4. No time-of-day differences on the acute effect of moderate-intensity exercise on IL-6 in men and women.

We observed in women an immediate increase in IL-6 post-exercise and subsequent decrease at 90 min post-exercise (P time = 0.004) (Figure 22H), but not in men (P time = 0.038) (Figure 22D). However, the acute response of IL-6 to the exercise protocol was similar in the morning and the evening (all P time x condition > 0.4) in both men (Figure 22D) and women (Figure 22H).

4.5. Lac-Phe, lactate and IL-6 could modulate appetite feelings after moderate-intensity aerobic exercise in women with influence of the time-of-day

We further conducted simple linear regressions to investigate the potential relationship of Lac-Phe, lactate and IL6 with appetite feelings' ratings at post and 90 min post exercise. In men, Lac-Phe (Figure 23A-D), lactate (Figure 23E-H) or IL6 (Figure 24A-D) were not associated with appetite feelings in the morning or the evening (all P > 0.1). Contrary, in women we observed that the increased levels of Lac-Phe was related to an increase in fullness at post-exercise in the evening (Evening: R^2 = 0.24; β = 18.61; P = 0.045) but not the morning (Morning: R^2 = 0.04; β = 6.81; P = 0.45) (Figure 23O). Similar results were obtained for lactate and fullness post-exercise (Morning: R^2 = 0.19; β = 1.61; P = 0.079; Evening: R^2 = 0.41; β = 1.57; P = 0.006) (Figure 23U), and IL-6 and hunger post-exercise (Morning: R^2 = 0.03; β = 6.93; P = 0.517; Evening: R^2 = 0.32; β = 27.53; P = 0.022) (Figure 24E). At 90 min post-exercise, lactate and hunger (Morning: R^2 = 0.19; β = -3.86; P = 0.078); Evening: R^2 = 0.52; β = -2.22; P = 0.003) (Figure 23T), and lactate and fullness (Morning: R^2 = 0.00; β = -0.10; P = 0.94; Evening: R^2 = 0.51; β = 1.84; P =0.003) (Figure 23V) were significantly related only in the evening.

The present results suggest that in women, the exercise-induced Lac-Phe, lactate and IL-6 may play a role at modulating the immediate appetite responses to moderate-intensity aerobic exercise after evening but not morning exercise.

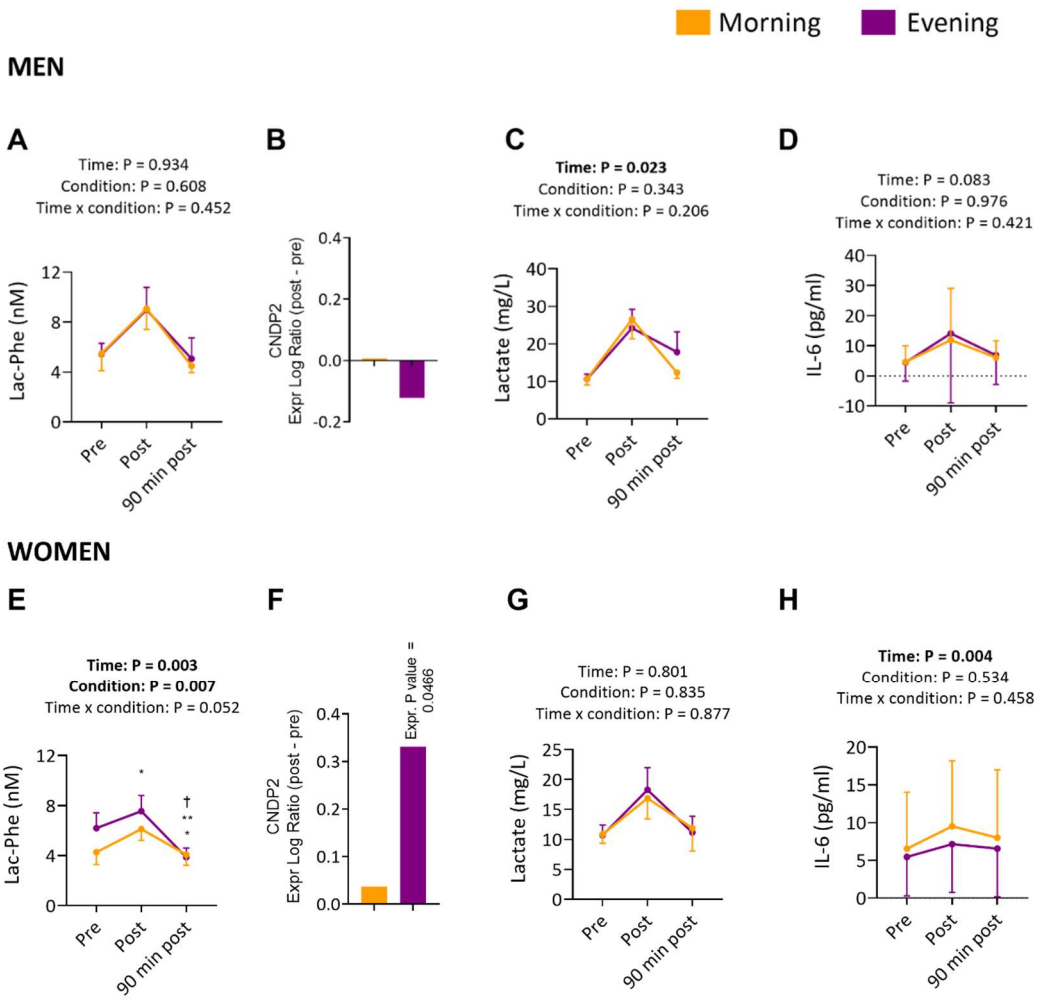


Figure 22. Lac-Phe, CNDP2, lactate and IL6 response to moderate-intensity aerobic exercise in men and women. Plasma Lac-phe at pre, post and 90 min post-exercise in the morning and evening conditions in men (A) and women (E). Change in the expression level in the skeletal muscle from pre to post exercise of the gen CNDP2 in the morning and evening in men (B) and women (F). Plasma lactate at pre, post and 90 min post-exercise in the morning and evening conditions in men (C) and women (G). Serum IL-6 at pre, post and 90 min post-exercise in the morning and evening conditions in men (H) and women (I). Using normal mixed models considering time a continuous variable (time, condition and time and condition [time x condition] interaction). Using normal mixed models considering time a categorical variable (pre vs. post and pre vs. 90 min post) for post-hoc analyses: * time = P < 0.05; ** time = P < 0.01; *** time = P < 0.001; † condition x time = P < 0.05. Values are mean and CI.

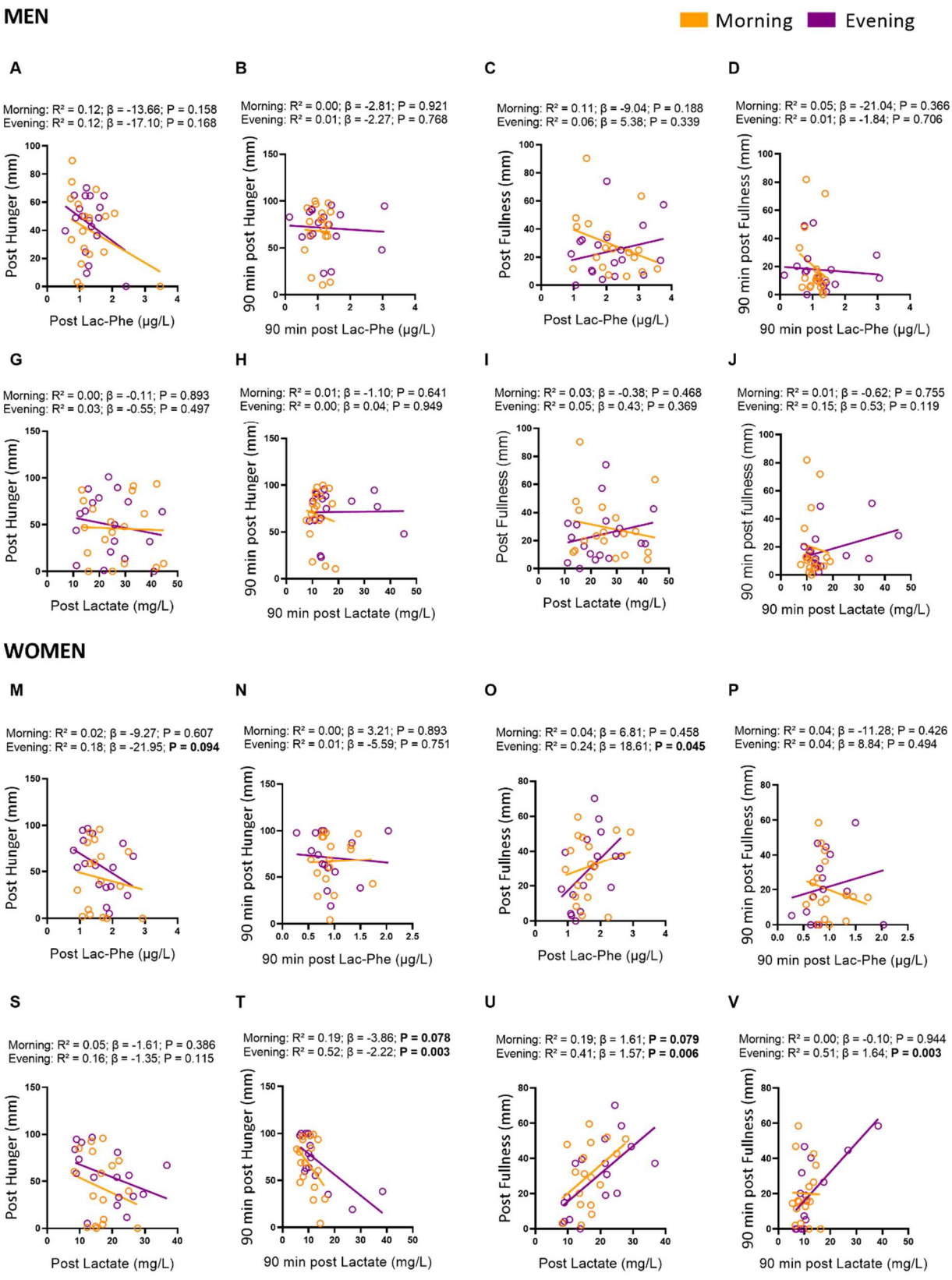


Figure 23. Correlations between Lac-Phe and Lac and main appetite feeling after moderate-intensity aerobic exercise in men and women. Correlation between Lac-Phe and hunger at post (A) and 90 min post (B) in men, and women (M, N). Correlation between Lac-Phe and fullness at post (C) and 90 min post (D) in men, and women (O, P). Correlation between lactate and hunger at post (G) and 90 min post (H) in men, and women (S, T). Correlation between lactate and fullness at post (I) and 90 min post (J) in men, and women (U, V). Using simple lineal regression.

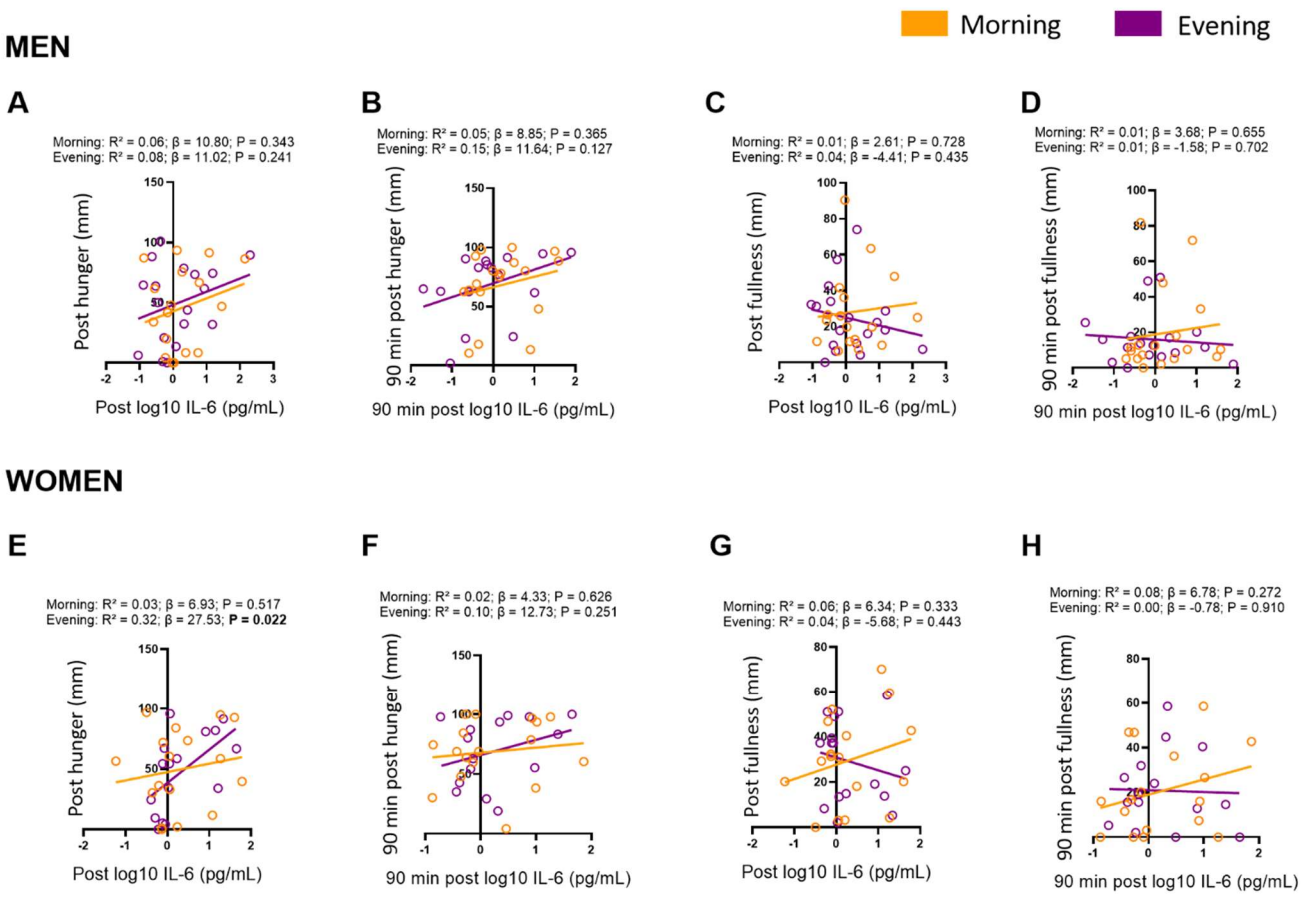


Figure 24. Correlations between IL6 and main appetite feeling after moderate-intensity aerobic exercise in men and women. Correlation between IL6 and hunger at post (A) and 90 min post (B) in men, and women (E, F). Correlation between IL6 and fullness at post (C) and 90 min post (D) in men, and women (G, H). Using simple lineal regression.

5. Discussion

The present study shows that a single bout of moderate-intensity aerobic exercise impact on appetite feelings, Lac-Phe, lactate and IL-6 with no influence of the time-of-day for both men and women. However, evening moderate-intensity aerobic exercise reveals a heightened potential to impact appetite in women. This is attributed to an increased expression of CNDP2 in the skeletal muscle and a significant relationship between appetite feelings and levels of Lac-Phe, lactate and IL-6 exclusively at this time of day. In men, the same exercise protocol does not provide a sufficient stimulus to impact appetite regulation, either in the morning or evening.

Consistent with other crossover studies[12,13], the response of appetite feelings to moderate-intensity aerobic exercise was similar in the morning and evening, for both healthy men

and women. This may be because our exercise protocol was not intense enough, which could also explain why we did not observe a suppression of hunger. Nevertheless, time-of-day differences on appetite feelings has neither been observed in men after performing a single bout high intensity interval exercise, despite lower levels of ghrelin in the afternoon[12]. The available evidence suggests that there is not a diurnal variation in the acute response of appetite to exercise. However, the exercise-induced response remains unexplored in women undergoing high-intensity exercise and individuals with obesity, highlighting a crucial gap in our understanding that demands further investigation. We could anticipate different results in patients with overweight or obesity, as evidenced by the study from Alizadeh et al.,[14]. They observed increased satiety following morning aerobic moderate-intensity exercise in women with overweight, but not in the afternoon. It is important to note that the design and statistical analysis of their study are not directly comparable to ours or to those previously mentioned.

Understanding the role of myokines and underlying mechanisms may help to predict potential differences on the acute and long-term effect of exercise on appetite, depending on the sex and time-of-day. Recently, Lac-Phe (synthetized from lactate by the cytosolic enzyme CNDP2) has been identified as possibly the most significant exercise-induced metabolite, playing a determinant role on appetite suppression[21]. To the best of our knowledge, this is the first study investigating Lac-Phe response to exercise in men and women and additionally considering the time-of-day. We found that CNDP2 expression in the skeletal muscle was significantly increased after exercise exclusively in women in the evening condition. This finding is of great interest and confirms that exercising at the right time-of-day can provide optimal benefits. It should be noted that in women in the evening, a negative feedback mechanism appears to occur, where the increase in CNDP2 post-exercise is followed by a drop in Lac-Phe below baseline levels at 90 minutes post-exercise. Lac-Phe was positively related to fullness only at post-exercise, suggesting that its effect on appetite dissipates by 90 minutes post-exercise. Presumably, a protocol of higher intensity would exert longer duration effects [21], and could translate to significant time-of-day differences on Lac-Phe. We cannot determine if the reported sex and time-of-day impact on CNDP2, Lac-Phe and its relation to fullness after exercise would result in decreased energy intake. Acute Lac-Phe treatment (circulating concentration of ~175 µM) suppressed food intake in mice [21]; however, this concentration is not comparable to the levels in plasma of the participants in this study (~6 to ~9 µM). Moreover, the only study investigating the effect of exercise on Lac-Phe, appetite feelings and energy intake did not

observe decreased energy intake despite increased Lac-Phe and decreased hunger after moderate intensity exercise with blood flow restriction [23].

Exercise intensity is pivotal for inducing appetite suppression[3] and may partially explain the sex and time-of-day differences we observe in this study. Using predominantly high-intensity protocols, other studies have reported that evening exercise is more effective for increasing lactate or IL-6 in healthy men[29–31]. However, exercise intensity in our study was insufficient to impact appetite outcomes in men, either in the morning or evening. Although lactate levels transiently increased after exercise, the lack of CNDP2 expression prevented its conversion to Lac-Phe. Further indicating an insufficient stimulus, there were no morning or evening changes in IL-6 induced by exercise. In coherence, we observed no significant association between Lac-Phe, lactate or IL-6 and appetite feelings at any time-of-day in men. Contrary, exercise intensity was enough in women to convert Lac-Phe from lactate and to increase IL-6 independently of the time of day. In the female's skeletal muscle, the gene expression of IL-6 in occurred only the evening. Additionally, pathways linked to inflammation were overall further activated in in the skeletal muscle in women than men. More specifically, they were further activated in the evening in women (data under review). Results from the simple linear regressions also suggested a role of lactate and IL-6 on modulating appetite feelings after exercise only in the evening in women.

Based on the gathered information, we postulate that acute evening exercise could be more effective than morning for appetite suppression in women. The effect may be achieved with moderate to high-intensity aerobic exercise, while a higher intensity may be necessary to observe time-of-day differences in men. Whether evening exercise would lead to decreased energy intake compared to morning remains to be determined.

Of note is that our study presents several limitations. First, the moderate-intensity exercise employed may not adequately reveal potential differences in acute appetite responses between morning and evening sessions. Additionally, while fasting hours and previous meals were standardized in both conditions, participants consumed one meal before the morning session and two meals before the evening session. Finally, the results cannot be extrapolated to energy intake or gut hormone responses due to the absence of these measurements.

6. Conclusion

Evening moderate-intensity aerobic exercise could be more effective for appetite suppression than morning exercise in healthy women. Although the impact of a single bout of moderate-intensity aerobic exercise on appetite feelings, Lac-Phe, lactate, and IL-6 was similar regardless of time-of-day, CNDP2 expression in skeletal muscle were induced exclusively after evening exercise in women. Also, Lac-Phe, lactate, and IL-6 were linked to appetite feelings only in the evening in women. Regardless the time-of-day, this exercise protocol had minimal impact on appetite regulation in healthy men. Future studies should determine the response with higher intensity exercise protocols, the long-term effect and the response in individuals with obesity.

7. References

1. Bray GA, Kim KK, Wilding JPH. Obesity: a chronic relapsing progressive disease process. A position statement of the World Obesity Federation. Obesity Reviews 2017; 18(7):715–723.

2. Hall KD. Did the Food Environment Cause the Obesity Epidemic? Obesity 2018; 26(1):11–13.

3. Thackray AE, Stensel DJ. The impact of acute exercise on appetite control: Current insights and future perspectives. Appetite 2023; 186:106557.

4. Blundell JE, Gibbons C, Caudwell P, et al. Appetite control and energy balance: impact of exercise. Obesity Reviews 2015; 16(S1):67–76.

5. Beaulieu K, Oustric P, Finlayson G. The Impact of Physical Activity on Food Reward: Review and Conceptual Synthesis of Evidence from Observational, Acute, and Chronic Exercise Training Studies. Curr Obes Rep 2020; 9(2):63–80.

6. Westerterp KR, Klaas *, Westerterp R, et al. Exercise, energy balance and body composition. European Journal of Clinical Nutrition 2018 72:9 2018; 72(9):1246–1250.

7. King NA, Hopkins M, Caudwell P, et al. Individual variability following 12 weeks of supervised exercise: identification and characterization of compensation for exercise-induced weight loss. Int J Obes 2008; 32(1):177–184.

8. Koronowski KB, Sassone-Corsi P. Communicating clocks shape circadian homeostasis. Science (1979) 2021; 371(6530).

9. Willis EA, Creasy SA, Honas JJ, et al. The effects of exercise session timing on weight loss and components of energy balance: midwest exercise trial 2. Int J Obes 2020; 44(1):114–124.

10. Alizadeh Z, Younespour S, Rajabian Tabesh M, et al. Comparison between the effect of 6 weeks of morning or evening aerobic exercise on appetite and anthropometric indices: a randomized controlled trial. Clin Obes 2017; 7(3):157–165.

11. Di Blasio A, Di Donato F, Mastrodicasa M, et al. Effects of the time of day of walking on dietary behaviour, body composition and aerobic fitness in post-menopausal women. J Sports Med Phys Fitness 2010; 50(2):196-201.

12. Larsen P, Marino F, Melehan K, et al. Evening high-intensity interval exercise does not disrupt sleep or alter energy intake despite changes in acylated ghrelin in middle-aged men. Exp Physiol 2019; 104(6):826–836.

13. Maraki M, Tsofliou F, Pitsiladis YP, et al. Acute effects of a single exercise class on appetite, energy intake and mood. Is there a time of day effect? Eur J Transl Myol 2023; 33(2):11104

14. Alizadeh Z, Mostafaee M, Mazaheri R, et al. Acute effect of morning and afternoon aerobic exercise on appetite of overweight women. Asian J Sports Med 2015; 6(2):1–6.

15. Veronda AC, Kline CE, Irish LA. The impact of circadian timing on energy balance: an extension of the energy balance model. Health Psychol Rev 2022; 16(2):161–203.

16. McCarthy SF, Islam H, Hazell TJ. The emerging role of lactate as a mediator of exercise-induced appetite suppression. American Journal of Physiology-Endocrinology and Metabolism 2020; 319(4):E814–E819.

17. Islam H, Townsend LK, McKie GL, et al. Potential involvement of lactate and interleukin-6 in the appetite-regulatory hormonal response to an acute exercise bout. J Appl Physiol 2017; 123(3):614–623.

18. Schultes B, Schmid SM, Wilms B, et al. Lactate infusion during euglycemia but not hypoglycemia reduces subsequent food intake in healthy men. Appetite 2012; 58(3):818–821.

19. Almada C, Cataldo LR, Smalley S V., et al. Plasma levels of interleukin-6 and interleukin-18 after an acute physical exercise: relation with post-exercise energy intake in twins. J Physiol Biochem 2013; 69(1):85–95.

20. Engelstoft MS, Park W mee, Sakata I, et al. Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. Mol Metab 2013; 2(4):376–392.

21. Li VL, He Y, Contrepois K, et al. An exercise-inducible metabolite that suppresses feeding and obesity. Nature 2022 606:7915 2022; 606(7915):785–790.

22. Lund J, Clemmensen C, Schwartz TW. Outrunning obesity with Lac-Phe? Cell Metab 2022; 34(8):1085–1087.

23. Li S, Guo R, Wang J, et al. The effect of blood flow restriction exercise on N-lactoylphenylalanine and appetite regulation in obese adults: a cross-design study. Front Endocrinol (Lausanne) 2023; 14:1289574.

24. Hoene M, Zhao X, Machann J, et al. Exercise-Induced N-Lactoylphenylalanine Predicts Adipose Tissue Loss during Endurance Training in Overweight and Obese Humans. Metabolites 2023; 13(1).

25. Shuhart CR, Yeap SS, Anderson PA, et al. Executive Summary of the 2019 ISCD Position Development Conference on Monitoring Treatment, DXA Cross-calibration and Least Significant Change, Spinal Cord Injury, Peri-prosthetic and Orthopedic Bone Health, Transgender Medicine, and Pediatrics. Journal of Clinical Densitometry 2019; 22(4):453–471.

26. Amaro-Gahete FJ, Sanchez-Delgado G, Jurado-Fasoli L, et al. Assessment of maximal fat oxidation during exercise: A systematic review. Scand J Med Sci Sports 2019; 29(7):910–921.

27. Midgley AW, Mcnaughton LR, Polman R, et al. Criteria for Determination of Maximal Oxygen Uptake A Brief Critique and Recommendations for Future Research. Sports Med 2007; 37(12):1019–1028.

28. Zhao X, Zeng Z, Chen A, et al. Comprehensive Strategy to Construct In-House Database for Accurate and Batch Identification of Small Molecular Metabolites. Anal Chem 2018; 90(12):7635–7643.

29. Souissi A, Yousfi N, Souissi N, et al. The effect of diurnal variation on the performance of exhaustive continuous and alternated-intensity cycling exercises. PLoS One 2020; 15(12):e0244191.

30. Hammouda O, Chtourou H, Chaouachi A, et al. Time-of-day effects on biochemical responses to soccer-specific endurance in elite Tunisian football players. J Sports Sci 2013; 31(9):963–971.

31. Abedelmalek S, Chtourou H, Aloui A, et al. Effect of time of day and partial sleep deprivation on plasma concentrations of IL-6 during a short-term maximal performance. Eur J Appl Physiol 2013; 113(1):241–248.

GENERAL DISCUSSION

Chapter 6. An integrative discussion of the International Doctoral Thesis

Mammalian cells possess molecular clocks that regulate whole-body metabolism[1]. These clocks are intricately related to cardiometabolic health, being its disruption linked to health disorders such as cardiovascular disease, type 2 diabetes, or obesity[2]. Exercise, a well-recognizedº ally in maintaining overall health, also acts as a zeitgeber and has the potential to reset molecular clocks[3]. This International Doctoral Thesis aimed to study the acute morning vs. evening impact on the effect of exercise on glucose regulation and energy metabolism in men and women, additionally unveiling the underlying molecular mechanisms in the skeletal muscle.

1. Findings in summary

Overall, the time-of-day of a single bout of moderate-intensity exercise does not influence the systemic effects on blood pressure, glucose regulation, energy metabolism and perceived appetite feelings. However, the molecular changes in the skeletal muscle after moderate-intensity aerobic exercise show an evident sexual dimorphism and time-of-day differences. In women evening exercise results a stronger stimulus than morning, with higher reliance on carbohydrates in the skeletal muscle, increased cytokine expression, and increased potential to suppress appetite in the short-term. Contrary, in the evening the molecular response in men promotes mitochondrial function while inhibiting carbohydrate utilization, although time-of-day variations are less evident than in women. These findings highlight the importance of considering sex-specific differences when designing exercise interventions. A single bout of moderate-intensity exercise was not enough to extrapolate the sex and time-of-day-dependent molecular changes to systemic effects. Therefore, questions remain unanswered regarding the effect of other exercise protocols (e.g., higher intensity or resistance training) and the response on patients with cardiometabolic disorders. Here, we provide a detailed molecular framework helpful for future research on precise exercise prescriptions in clinical settings.

2. Previous literature on the diurnal variation of the effect of a single bout of exercise on cardiometabolic health parameters in humans

To understand whether the effect of exercise on cardiometabolic health parameters differs when it is performed in the morning vs. the evening, it is essential first to define the acute physiological responses to exercise. To this end, we systematically reviewed and meta-analyzed the results of the available studies to date that investigated the morning vs.

evening impact of a single bout of exercise on blood pressure, blood glucose and blood lipids (Chapter 3). Results from this meta-analysis indicated that there was no influence of the time of the day on the effect of a single bout of exercise on blood pressure[4–13] and blood glucose[14–23], while the research on lipids was insufficient[24]. Specifically, both morning and evening exercise produced an acute hypotensive response and an increase in blood glucose. The main limitation we faced in this study was the notable qualitative differences between the small number of studies. Participants differed in age, training and health status, BMI, and sex. Exercise protocols also varied in type, intensity, and duration. We acknowledged a critical gap in female research since only 27.3% of the meta-analysed studies included women[4,8,17,18,20,21], what hampered to determine potential specific responses based on biological sex[25]. After detailed revision, we concluded that also the health status probably interfered with the morning vs. evening exercise effects. Most studies on healthy participants reported no time-of-day differences in the blood pressure and blood glucose response to exercise[5,6,8,10–13]. In contrast, studies on hypertensive patients did, although they were contradictory between each other[7,9]. Additionally, only studies conducted in patients with diabetes mellitus described increased blood glucose after morning exercise that did not occur in response to evening exercise [16,18,21]. Despite these descriptive differences, we did not find statistical significance for the moderation of participants' age, health status, BMI, sex, or exercise intensity and duration on results. Still, we believe it could be due to the limited number of studies included in each moderator level, and in the case of sex, the reduced number of women participating in the studies.

3. Diurnal variation of the effect of a single bout of moderate-intensity aerobic exercise on cardiometabolic health parameters in humans: role of sex

Largely due to the underrepresentation of women in the available literature to date, drawing robust conclusions about the acute effects effect of the time-of-day of exercise on cardiometabolic health parameters was challenging. To address the sex gap, we developed a study aiming to elucidate the physiological and molecular mechanisms underlying potential sex-specific time-of-day variations in exercise-induced glucose regulation and energy metabolism (Chapter 4). The study revealed that a single bout of moderate-intensity aerobic exercise, whether performed in the morning or evening, elicited similar systemic effects on glucose regulation and energy metabolism in both men and women. Remarkably, there existed a pronounced sexual dimorphism and time-of-day variations in the skeletal muscle adaptations at the molecular level.

Glycaemic metabolism. Confirming the results from our previous meta-analysis, in Chapter 4 we observed that the time-of-day did not impact on systemic glucose response to exercise in healthy population. At the systemic level, biological sex differences were not evident. Our findings in women were consistent with those of Galliven et al., even though they employed a higher intensity protocol of running for 20 minutes at 90% VO_2max[20]. Similarly, in healthy men, no time-of-day differences were observed with moderate or high-intensity protocols [14,15,19,22,23]. Glycaemic markers – improved basal glucose/insulin ratio, HOMA index, insulin and cortisol – neither displayed time-of-day variations in response to exercise. In agreement, other authors have reported not differentially altered insulin or HOMA index by morning or evening exercise[26]. At the molecular level, however, sex and time-of-day differences became evident. In women, the transcriptional analysis revealed a greater number of post-exercise changes in genes involved in glycogen and glucose degradation in the evening, with most of them being upregulated. In contrast, men exhibited fewer changes in these family of genes, and they were predominantly repressed following evening exercise.

Lipid metabolism. We did not identify time-of-day impact on post-exercise total-cholesterol or LDL-cholesterol. The response of serum triglycerides, however, suggested time-of-day variations in women. Triglycerides in females exhibited a reduction following evening but not morning exercise, showing proximity to statistical significance. This data concur with a previous study that demonstrated a decrement of triglycerides levels after evening vs. morning submaximal aerobic exercise in healthy men [24]. With our exercise protocol, however, we did not observe that distinction in men. As mentioned in Chapter 3, research on the time-of-day of exercise and the immediate response of blood lipids is scarce. Interestingly, a recent systematic review and meta-analyses comparing the metabolic adaptations after more than 2 weeks of morning and afternoon training has described afternoon as a more effective way for reducing circulating triglyceride levels, particularly if exercise is conducted at high intensity [26]. We cannot fully clarify whether there is a distinct response between men and women because this review does not consider sex differences. Nevertheless, we postulate that a higher exercise intensity is required to observe a diurnal variation of lipid levels in men compared to women.

Energy metabolism. In terms of substrate utilization, previous research had suggested a sexual dimorphism and time-of-day variations in fat oxidation rates[27,28]. Men showed higher lipid utilization in the evening in comparison to morning when exercising at maximal fat oxidation intensity or low-moderate intensity[28–31]. Morning and evening

exercise, in contrast, did not lead to distinct effects in women at the intensity of maximal fat oxidation[27]. Regarding adaptations in the skeletal muscle, investigations conducted in men have suggested that afternoon training promotes fatty acids as substrate during exercise by enhancing mitochondrial oxidative capacity and lipid biosynthesis[32,33]. In Chapter 4 we did not achieve to demonstrate a diurnal variation on fat or carbohydrates oxidation (approached by RER) neither in men nor women. EE was also similar in both conditions and sexes in agreement with comparable studies[34,35]. Upon detailed examination, molecular changes suggest a differential effect of exercise on energy metabolism influenced by the time-of-day and sex. The mitochondrial proteome was particularly increased in the evening in women, while particularly decreased at this time-of-day in men. Mitochondrial respiration showed substantial differences exclusively in women (showing proximity to statistical significance): respiration through complex II increased in the morning and decreased in the evening following exercise. The higher activity of complex II in the morning could result at first contradictory considering that females presented decreased mitochondrial proteome at this moment. We assume that a negative feedback loop regulation was occurring, wherein protein synthesis was downregulated in response to already increased mitochondrial function[36]. In men, the decreased mitochondrial proteome in the evening suggests an increased mitochondrial function at this moment what would align previous research[32,33]. Even so, the diurnal variation in the molecular adaptations of the skeletal muscle after exercise was more evident in women than men. Women's metabolism after morning moderate-intensity aerobic exercise appeared to depend more on fat oxidation than after evening exercise, while the opposite pattern could be occurring in men. It is plausible that time-of-day and sex variations in exercise response were partially defined by exercise intensity. More evident differences in men could be expected when high intensity exercise protocols would be applied[26].

Skin body temperature provided further information to understand dissimilarities based on sex and time-of-day. Biological sex differences in core body temperature, together with other circadian biomarkers such as melatonin and cortisol exist[37]. Women typically exhibit an earlier phase and a shorter circadian period in comparison to men[38,39]. We noticed that values from men's skin body temperatures at the starting of exercise in the evening were not significantly different compared to those obtained in the morning. On the opposite, women exhibited significantly higher skin body temperature in the evening at this point, a fact that would signal their shorter circadian period and the phase shift from diurnal to

nocturnal, giving an additional explanation to why our results displayed more evident time-of-day differences in women and opposite patterns between sexes. In agreement, higher temperature values would be expected to stimulate carbohydrate metabolism[40] in women during the evening, what in effect was evidenced by the female muscle's transcriptome.

One study had previously investigated the time-of-day of exercise effect on sleep quality. They concluded that morning, afternoon, and evening high intensity cycling had similar impact on most sleep variables with minor impacts observed on sleep quality following evening exercise[15]. We observed comparable results in Chapter 4, except from women that slept for longer after evening compared to the morning condition. Again, acute moderate-intensity exercise was more effective for elucidating time-of-day variations in women than men.

Men and women showed differentially expressed key myokines involved in energy metabolism homeostasis including both FGF21 and GDF15. FGF21 was only expressed in men in the post-evening exercise, while GDF15 was exclusively induced in women, with higher levels in the morning condition. Considering that these myokines regulate mitochondrial function and particularly lipid metabolism[41–43], the results support that women presented a more oxidative metabolism when exercising in the morning. Additionally, the higher cytokine expression observed in women following evening exercise suggested the same exercise protocol was representing a more intense stimulus in the evening for them leading to increased expression genes involved in carbohydrate metabolism[44]. The exclusive expression of FGF21 after the evening exercise in men supported increased mitochondrial function and more oxidative metabolism, data that concur with previous works[28–33]. However, no significant time-of-day differences in cytokine expression were observed in men.

Differences between sexes and time-of-day in energy metabolism could logically extend to a different impact of exercise on appetite regulation. In Chapter 5, we examined the acute impact of morning vs. evening moderate intensity exercise on appetite-related outcomes in healthy men and women. Exercise similarly affected appetite feelings—hunger, fullness, craving for tasty, and craving for sweet—in response to morning and evening exercise protocols for both sexes. Comparable crossover studies have reported no differences in appetite sensations or energy intake based on the time-of-day neither in healthy women after 60 minutes of aerobic exercise[45], or in healthy men after 30 minutes of high-intensity interval training despite lower post-exercise ghrelin levels in the

afternoon [15]. Consequently, the time-of-day does not appear to significantly influence appetite responses to acute exercise. However, the limited number of studies on this topic leaves certain questions unanswered, such as the time-of-day potential effects after high-intensity exercise in women.

Exercise intensity is pivotal for inducing appetite suppression. This phenomenon is primarily explained by the action of intensity-dependent energy regulatory myokines, such as IL-6, lactate, and Lac-Phe, which mediate changes in the gut, adipose tissue, and central nervous system[46–48] . The intensity of exercise in our study was insufficient to decrease hunger or increase fullness independently of the time of day. However, higher cytokine expression in women than men supported that adaptations in the females' skeletal muscle were more similar to those typically expected from high intensity exercise. In accordance, we observed that exercise intensity was enough in women to convert lactate to Lac-Phe and to increase IL-6 independently of the time of day, however it did not occur in men. Mainly in response to high-intensity exercise, comparable studies have demonstrated time of day differences on lactate and IL-6 in men favoring evening[49–52]. Once more, there is limited research on women [20].

Based on this previous literature and our comprehensive dataset, we believe that exercise could be more effective for appetite suppression in the evening than in the morning, and this could be achieved in women with moderate to high intensity exercise, while men would benefit from exercising at higher intensity. In this context, Lac-Phe is of particular interest. Lac-Phe has been identified to be key for suppressing food intake and it is one of the most significant metabolites induced by exercise[48]. To the best of our knowledge, our study is the first to investigate sex and time of day differences on exercise-induced Lac-Phe and the enzyme responsible of its synthesis, CNDP2. We observed a significant expression of CNDP2 post-exercise only in the evening women. Moreover, we detected significant relationship between Lac-Phe, lactate and IL-6 and appetite feelings exclusively at that time-of-day. In contrast, moderate aerobic exercise was insufficient to significantly increase the expression of CNDP2 in men or demonstrate relationship between the myokines and appetite feelings after exercise.

Of note, these are exploratory results, and we can not conclude that time-of-day and sex differences would translate to reduced energy intake. The increase in CNDP2 expression post-evening exercise in women was followed by a drop in Lac-Phe below baseline levels what suggested a negative regulatory feedback mechanism. Lac-Phe and appetite feelings

were no longer related at 90 min post-exercise. Therefore, the response was transitory. Levels of circulating Lac-Phe in our participants (from ~6 to ~9 nM) were very low compared to those required for reducing energy intake in mice (~175 µM)[48]. To date, the only study investigating the effect of exercise on Lac-Phe, appetite feelings, and energy intake in humans did not report a decreased energy intake despite increased Lac-Phe and reduced hunger after moderate-intensity exercise with blood flow restriction[53]. It is probable that moderate-intensity aerobic exercise is enough to demonstrate time of day differences in women at the molecular level in terms of appetite regulation, but not to translate them to acute changes on energy intake.

Some interrogations remain to be solved. The myokine GDF15 (which has been associated with appetite suppression[54]) was upregulated more prominently in the morning in women. Also, we observed a positive relation between post-evening exercise IL-6 and hunger in women. How would the time-of-day of exercise and sex impact on energy intake and gut hormones is still uncertain.

4. Diurnal variation of the effect of a single bout of exercise on cardiometabolic health parameters in humans: potential role of health status

Results from Chapter 4 are limited to healthy population. However, according to the results provided in Chapter 3, the impact of the time-of-day of exercise on cardiometabolic health may be probably influenced by health status. Findings from the meta-analysis revealed similar hypotensive effects of exercise independently of the time-of-day, which was typically observed on studies conducted in healthy men[5–8,10,13] . In contrast, literature showed controversy when it came to pre-hypertensive or hypertensive men[7,9]. According to Brito et al., morning exercise was more effective for lowering blood pressure in prehypertensive men[9], but evening exercise was better in hypertensive men receiving ARB medication[7]. No time-of-day differences were reported in hypertensive women[4] or hypertensive men taking ACEi medication[7]. A more recent systematic review has concluded that evening exercise may provide better clinical outcomes in patients with hypertension[55]. The greater reduction of blood triglycerides following afternoon/evening exercise has been proposed to be linked to this improvement [26].

Blood glucose showed no significant variations after a single bout of morning or evening exercise, according to our meta-analysis in Chapter 3. However, there was a trend suggesting that morning exercise might have a more substantial impact on glucose levels compared to evening exercise. Although the health status did not significantly moderate

the blood glucose response between morning and evening exercise, only studies involving participants with diabetes mellitus reported time-of-day differences in glucose response. These works typically indicated an increase in blood glucose following morning exercise, a result that was not observed after evening exercise[16,18,21]. Increasing evidence suggests that morning exercise may be less advisable for patients with diabetes mellitus. Results from a prospective cohort study reported reduced insulin resistance in participants most active in the afternoon or evening, but not in those most active in the morning[56]. Long-term interventions also have described that evening exercise helps to prevent hyperglycemic events in patients with type 2 diabetes[26,57]. Additionally, a study conducted in men with overweight/obesity reported improvements in glycaemic control and partial reversal of high fat diet-induced changes in metabolic profiles only when participants exercised in the evening vs. the morning[58]. In contrast, an exercise intervention in middle-aged adults with metabolic syndrome showed that morning exercise improved fasting insulin levels to a greater extent than afternoon exercise, although no differences were noted in fasting glucose levels between morning and afternoon exercise[59]. Therefore, prescribing morning or evening exercise may depend on the patient's condition. For healthy population, according to results from Chapter 3 and Chapter 4, the time-of-day of exercise may not significantly impact systemic glucose levels.

It is important to note that none of the mentioned long-term studies analyzed the data for men and women separately, which hampers the determination of health and sex-specific exercise-induced effects. The mechanism underlying improved benefits from afternoon/evening training are possibly explained by a preservation of muscle and hepatic glycogen to favor lipid oxidation[60]. From the transcriptomic results in Chapter 4, it seems evident that men preserved glycogen in the evening, while women did not. Because of their earlier phase shift [38,39], we theorize that female patients with type 2 diabetes may benefit from exercising in the afternoon rather than the evening. Consistently, in the study from Arciero et al. (i.e., 12-week multimodal exercise intervention at 6:00 vs. 18:30), women in the morning group experienced greater abdominal fat loss and blood pressure reduction, while men in the evening group increased their fat oxidation capacity and reduced their systolic blood pressure and fatigue[25].

It has been easier to find long-term studies focused on women when they explore fat and weight loss. Still, literature is conflicting. A recent study involving women with overweight/obesity found that 12 weeks of self-paced aerobic exercise in the afternoon led to slightly greater weight loss than morning exercise[61]. These results concur with those

obtained in another study performed in post-menopausal women who walked for 3 months (50 mins at 55% HRreserve, 4 days/week) and obtained increased fat loss in the evening group – scheduled before dinner – compared to the morning group – scheduled after breakfast –[62]. Conversely, other studies which included both men and women with overweight/obesity indicated that moderate aerobic training in the morning was more effective for reducing fat mass and weight than afternoon training[63,64]. Most investigations agreed that energy intake and appetite feelings were not differently affected by the time-of-day[61–63]. However, a notable aspect among the mentioned studies is the timing of meals relative to exercise. Most studies allowed flexible meal schedules[61–63] excepting from the study by Di Blasio et al. that implemented the dinner after the walking activity [62]. Women in the evening group did not decrease their total energy intake compared to those in the morning group, but shifted their intake towards the morning and experienced greater fat loss. In line with our results from Chapter 5, we could expect diurnal variations in the effect of exercise scheduled before a meal. However, long-term metabolic adaptations after morning vs. afternoon/evening exercise implemented before a meal has not yet been investigated.

In the scenario of exercise as medicine, afternoon or evening training appears to be more effective for lowering blood pressure and preventing hypoglycemia in type 2 diabetes patients. For appetite regulation, weight, and fat loss, the results are more controversial due to the unclear interaction between the time-of-day of exercise and the timing of meals relative to exercise. This International Doctoral Thesis contributes to the field by helping to design more precise and inclusive exercise interventions and guidelines. Anyhow, exercise remains an excellent preventative and therapeutic method for improving cardiometabolic health, including blood pressure, insulin sensitivity, glucose tolerance, lipid profile, and appetite regardless of the time-of-day[65,66].

5. Limitations

This International Doctoral Thesis has several limitations that should be acknowledge. In Chapter 3, the analysis of the acute effects of the time-of-day on exercise involved 11 studies for systolic and diastolic blood pressure, 7 studies for mean blood pressure, and 10 studies for blood glucose. These works exhibited qualitative differences in exercise protocols, participant's health and training status, BMI, sex, and specific hours within morning or evening conditions. Although no statistical heterogeneity was found in the meta-analyses, it may be attributed to the small sample sizes of the included studies.

Moreover, we did not address the gap into potential sex differences in the acute effects of exercise timing on blood pressure.

In Chapter 4 and Chapter 5, one single bout of exercise may not suffice to observe systemic effects of the time-of-day of exercise. The results coming from a healthy population should be cautiously interpreted when applying them to patients with cardiometabolic disorders. Also, investigating the time-of-day is challenging, as morning and evening encompasses several possible hours. Even more the definitions of morning, afternoon, and evening vary across cultures, making hard the comparisons between studies.

Of particular interest for appetite outcomes in Chapter 5, despite standardizing fasting hours and prior meals, participants consumed one meal before the morning session and two before the evening session. These results are therefore exploratory, and the absence of energy intake or gut hormone measures limits the conclusions.

Lastly, although the menstrual phase of female participants was recorded, logistical constraints prevented to match morning and evening conditions within the same phases. While the effect of exercise on substrate oxidation or appetite outcomes does not significantly vary across the menstrual cycle[67–69], accounting for menstrual phases is considered crucial to avoid potential confounding variables[37]. Further, it has been reported that the diurnal variation of glucose response to exercise is influenced by the menstrual cycle [20].

6. References

1. Panda S, Hogenesch JB, Kay SA. Circadian rhythms from flies to human. Nature 2002; 417:329–335.

2. Panda S. Circadian physiology of metabolism. Science (1979) 2016; 354(6315):1008–1015.

3. Gabriel BM, Zierath JR. Circadian rhythms and exercise - re-setting the clock in metabolic disease. Nat Rev Endocrinol 2019; 15(4):197–206.

4. Azevêdo LM, Souza AC de, Santos LES, et al. Fractionated Concurrent Exercise throughout the Day Does Not Promote Acute Blood Pressure Benefits in Hypertensive Middle-aged Women. Front Cardiovasc Med 2017; 4:1–7.

5. Boukelia B, Gomes EC, Florida-James GD. Diurnal variation in physiological and immune responses to endurance sport in highly trained runners in a hot and humid environment. Oxid Med Cell Longev 2018; 2018.

6. Boussetta N, Abedelmalek S, Aloui K, Souissi N. The effect of air pollution on diurnal variation of performance in anaerobic tests, cardiovascular and hematological parameters, and blood gases on soccer players following the Yo–Yo Intermittent Recovery Test Level-1. Chronobiol Int 2017; 34(7):903–920.

7. Brito LC, Azevêdo L, Peçanha T, et al. Effects of ACEi and ARB on post-exercise hypotension induced by exercises conducted at different times of day in hypertensive men. Clin Exp Hypertens 2020; 42(8):722–727.

8. Chan-Dewar F, Gregson W, Whyte G, et al. Do the effects of high intensity 40 km cycling upon left ventricular function and cardiac biomarker during recovery vary with time of day? J Sports Sci 2013; 31(4):414–423.

9. De Brito LC, Rezende RA, Da Silva ND, et al. Post-exercise hypotension and its mechanisms differ after morning and evening exercise: A randomized crossover study. PLoS One 2015; 10(7):1–16.

10. Focht BC, Koltyn KF. Alterations in pain perception after resistance exercise performed in the morning and evening. 2009; 23(3):891–897.

11. Jones H, Pritchard C, George K, Edwards B, Atkinson G. The acute post-exercise response of blood pressure varies with time of day. Eur J Appl Physiol 2008; 104(3):481–489.

12. Jones H, Taylor CE, Lewis NCS, George K, Atkinson G. Post-exercise blood pressure reduction is greater following intermittent than continuous exercise and is influenced less by diurnal variation. Chronobiol Int 2009; 26(2):293–306.

13. O'Connor PJ, Davis JC. Psychobiologic responses to exercise at different times of day. Med Sci Sports Exerc 1992; 24(6):714–719.

14. McIver VJ, Mattin LR, Evans GH, Yau AMW. Diurnal influences of fasted and non-fasted brisk walking on gastric emptying rate, metabolic responses, and appetite in healthy males. Appetite 2019; 143:104411.

15. Larsen P, Marino F, Melehan K, Guelfi KJ, Duffield R, Skein M. Evening high-intensity interval exercise does not disrupt sleep or alter energy intake despite changes in acylated ghrelin in middle-aged men. Exp Physiol 2019; 104(6):826–836.

16. Savikj M, Gabriel BM, Alm PS, et al. Afternoon exercise is more efficacious than morning exercise at improving blood glucose levels in individuals with type 2 diabetes: a randomised crossover trial. Diabetologia 2019; 62(2):233–237.

17. Munan M, Dyck RA, Houlder S, et al. Does exercise timing affect 24-hour glucose concentrations in adults with type 2 diabetes? A follow up to the Exercise-Physical Activity and Diabetes glucose Monitoring Study. Can J Diabetes 2020; 44(8):711–718.

18. Toghi-Eshghi SR, Yardley JE. Morning (Fasting) vs Afternoon Resistance Exercise in Individuals with Type 1 Diabetes: A Randomized Crossover Study. Journal of Clinical Endocrinology and Metabolism 2019; 104(11):5217–5224.

19. Fernandes AL, Lopes-Silva JP, Bertuzzi R, et al. Effect of time of day on performance, hormonal and metabolic response during a 1000-M cycling time trial. PLoS One 2014; 9(10).

20. Galliven EA, Singh A, Michelson D, Bina S, Gold PW, Deuster PA. Hormonal and metabolic responses to exercise across time of day and menstrual cycle phase. J Appl Physiol 1997; 83(6):1822–1831.

21. Ruegemer, J., Squieres, Ray W., Marsh, Harold M., Haymond, Morey W., Cryer, Philip E., Rizza, Robert A., Miles JM. Differences Between Prebreakfast and Late Afternoon Lgycemic Responses to Exercise in IDDM Patients. J Chem Inf Model 2019; 53(9):1689–1699.

22. Hobson RM, Clapp EL, Watson P, Maughan RJ. Exercise capacity in the heat is greater in the morning than in the evening in man. Med Sci Sports Exerc 2009; 41(1):174–180.

23. Tanaka Y, Ogata H, Park I, et al. Effect of a single bout of morning or afternoon exercise on glucose fluctuation in young healthy men. Physiol Rep 2021; 9(7):e14784.

24. Hatice, Aldemir NK. The effect of time of day and exercise on platelet functions and platelet – neutrophil aggregates in healthy male subjects Hatice Aldemir and Nedret Kılı ̧. Mol Cell Biochem 2005; 280(1–2):119–124.

25. Arciero PJ, Ives SJ, Mohr AE, et al. Morning Exercise Reduces Abdominal Fat and Blood Pressure in Women; Evening Exercise Increases Muscular Performance in Women and Lowers Blood Pressure in Men. Front Physiol 2022; 13:893783.

26. Galan-Lopez P, Casuso RA. Metabolic Adaptations to Morning Versus Afternoon Training: A Systematic Review and Meta-analysis. Sports Medicine 123AD.

27. Robles-González L, Aguilar-Navarro M, López-Samanes Á, et al. No diurnal variation is present in maximal fat oxidation during exercise in young healthy women: A cross-over study. Eur J Sport Sci 2023; 23(6):936–942.

28. Amaro-Gahete FJ, Jurado-Fasoli L, Triviño AR, et al. Diurnal Variation of Maximal Fat-Oxidation Rate in Trained Male Athletes. Int J Sports Physiol Perform 2019; 14(8):1140–1146.

29. Ramírez-Maldonado M, Jurado-Fasoli L, del Coso J, R. Ruiz J, Amaro-Gahete FJ. Caffeine increases maximal fat oxidation during a graded exercise test: is there a diurnal variation? J Int Soc Sports Nutr 2021; 18(1):1–9.

30. Mohebbi H, Azizi M. Maximal fat oxidation at the different exercise intensity in obese and normal weight men in the morning and evening. J Hum Sport Exerc 2011; 6(1):49–58.

31. Sharma P, Agarwal M. Diurnal variation of fat oxidation rate and energy expenditure in an acute bout of endurance exercise by young healthy males. J Family Med Prim Care 2022; 11(1):240.

32. Ezagouri S, Zwighaft Z, Sobel J, Golik M, Verges S, Asher G. Physiological and Molecular Dissection of Daily Variance in Exercise Capacity Physiological and Molecular Dissection of Daily Variance in Exercise Capacity 2019:78–91.

33. Savikj M, Stocks B, Sato S, et al. Exercise timing influences multi-tissue metabolome and skeletal muscle proteome profiles in type 2 diabetic patients – A randomized crossover trial. Metabolism 2022; 135:155268.

34. Kim HK, Ando K, Tabata H, et al. Effects of Different Intensities of Endurance Exercise in Morning and Evening on the Lipid Metabolism Response. J Sports Sci Med 2016; 15(3):467.

35. Mode WJA, Slater T, Pinkney MG, et al. Effects of Morning Vs. Evening exercise on appetite, energy intake, performance and metabolism, in lean males and females. Appetite 2023; 182:106422.

36. Kummer E, Ban N. Mechanisms and regulation of protein synthesis in mitochondria. Nature Reviews Molecular Cell Biology 2021 22:5 2021; 22(5):307–325.

37. Lok R, Qian J, Chellappa SL. Sex differences in sleep, circadian rhythms, and metabolism: Implications for precision medicine. Sleep Med Rev 2024; 75:101926.

38. Cain SW, Dennison CF, Zeitzer JM, et al. Sex Differences in Phase Angle of Entrainment and Melatonin Amplitude in Humans. J Biol Rhythms 2010; 25(4):288–296.

39. Boivin DB, Shechter A, Boudreau P, Begum EA, Ng Ying-Kin NMK. Diurnal and circadian variation of sleep and alertness in men vs. naturally cycling women. Proceedings of the National Academy of Sciences 2016; 113(39):10980–10985.

40. Maunder ED, Plews DJ, Merien F, Kilding AE. Exercise intensity regulates the effect of heat stress on substrate oxidation rates during exercise. Eur J Sport Sci 2020; 20(7):935–943.

41. Chung HK, Ryu D, Kim KS, et al. Growth differentiation factor 15 is a myomitokine governing systemic energy homeostasis. J Cell Biol 2017; 216(1):149–165.

42. Felice V Di, Kumar A, Penna F, et al. FGF21 as Modulator of Metabolism in Health and Disease 2019; 10:419.

43. Trayhurn P, Drevon CA, Eckel J. Secreted proteins from adipose tissue and skeletal muscle – adipokines, myokines and adipose/muscle cross-talk. Arch Physiol Biochem 2011; 117(2):47–56.

44. Moldoveanu AI, Shephard RJ, Shek PN. The Cytokine Response to Physical Activity and Training. Sports Med 2001 Feb; 31(2):115–144.

45. Maraki M, Tsofliou F, Pitsiladis YP, Malkova D, Mutrie N, Higgins S. Acute effects of a single exercise class on appetite, energy intake and mood. Is there a time of day effect? Appetite 2005; 45(3):272–278.

46. Thackray AE, Stensel DJ. The impact of acute exercise on appetite control: Current insights and future perspectives. Appetite 2023; 186:106557.

47. Islam H, Townsend LK, McKie GL, Medeiros PJ, Gurd BJ, Hazell TJ. Potential involvement of lactate and interleukin-6 in the appetite-regulatory hormonal response to an acute exercise bout. J Appl Physiol 2017; 123(3):614–623.

48. Li VL, He Y, Contrepois K, et al. An exercise-inducible metabolite that suppresses feeding and obesity. Nature 2022 606:7915 2022; 606(7915):785–790.

49. Souissi A, Yousfi N, Souissi N, et al. The effect of diurnal variation on the performance of exhaustive continuous and alternated-intensity cycling exercises. PLoS One 2020; 15(12):e0244191.

50. Hammouda O, Chtourou H, Chaouachi A, et al. Time-of-day effects on biochemical responses to soccer-specific endurance in elite Tunisian football players. J Sports Sci 2013; 31(9):963–971.

51. Abedelmalek S, Chtourou H, Aloui A, Aouichaoui C, Souissi N, Tabka Z. Effect of time of day and partial sleep deprivation on plasma concentrations of IL-6 during a short-term maximal performance. Eur J Appl Physiol 2013; 113(1):241–248.

52. Kim HK, Konishi M, Takahashi M, et al. Effects of acute endurance exercise performed in the morning and evening on inflammatory cytokine and metabolic hormone responses. PLoS One 2015; 10(9):e0137567.

53. Li S, Guo R, Wang J, et al. The effect of blood flow restriction exercise on N-lactoylphenylalanine and appetite regulation in obese adults: a cross-design study. Front Endocrinol (Lausanne) 2023; 14:1289574.

54. Klein AB, Kleinert M, Richter EA, Clemmensen C. GDF15 in Appetite and Exercise: Essential Player or Coincidental Bystander? Endocrinology 2022; 163(1):bqab242.

55. Brito LC, Marin TC, Azevêdo L, Rosa-Silva JM, Shea SA, Thosar SS. Chronobiology of Exercise: Evaluating the Best Time to Exercise for Greater Cardiovascular and Metabolic Benefits. Compr Physiol 2022; 12(3):3621–3639.

56. Van Der Velde JHPM, Boone SC, Winters-Van Eekelen E, et al. Timing of physical activity in relation to liver fat content and insulin resistance H-MRS Proton magnetic resonance spectroscopy ISI Insulin sensitivity index LPA Light physical activity LUMC Leiden University Medical Center MET Metabolic equivalents of t. Diabetologia 2023; 66:461–471.

57. Teo SYM, Kanaley JA, Guelfi KJ, Marston KJ, Fairchild TJ. The Effect of Exercise Timing on Glycemic Control: A Randomized Clinical Trial. Med Sci Sports Exerc 2020; 52(2):323–334.

58. Moholdt T, Parr EB, Devlin BL, Debik J, Giskeødegård G, Hawley JA. The effect of morning vs evening exercise training on glycaemic control and serum metabolites in overweight/obese men: a randomised trial. Diabetologia 2021; 64(9):2061–2076.

59. Morales-Palomo F, Moreno-Cabañas A, Alvarez-Jimenez L, et al. Efficacy of morning versus afternoon aerobic exercise training on reducing metabolic syndrome components: A randomized controlled trial Key points. J Physiol 2023:1–15.

60. Casanova-Vallve N, Duglan D, Vaughan ME, et al. Daily running enhances molecular and physiological circadian rhythms in skeletal muscle. Mol Metab 2022; 61:101504.

61. Brooker PG, Gomersall SR, King NA, Leveritt MD. The efficacy of morning versus evening exercise for weight loss: A randomized controlled trial. Obesity 2023; 31(1):83–95.

62. Di Blasio A, Di Donato F, Mastrodicasa M, et al. Effects of the time of day of walking on dietary behaviour, body composition and aerobic fitness in post-menopausal women. J Sports Med Phys Fitness 2010; 50(2):196–201.

63. Willis EA, Creasy SA, Honas JJ, Melanson EL, Donnelly JE. The effects of exercise session timing on weight loss and components of energy balance: midwest exercise trial 2. Int J Obes 2020; 44(1):114–124.

64. Alizadeh Z, Younespour S, Rajabian Tabesh M, Haghravan S. Comparison between the effect of 6 weeks of morning or evening aerobic exercise on appetite and anthropometric indices: a randomized controlled trial. Clin Obes 2017; 7(3):157–165.

65. Gabriel BM, Zierath JR. The Limits of Exercise Physiology: From Performance to Health. Cell Metab 2017; 25(5):1000–1011.

66. Tian D, Meng J. Exercise for prevention and relief of cardiovascular disease: Prognoses, mechanisms, and approaches. Oxid Med Cell Longev 2019; 2019:3756750.

67. Horton TJ, Miller EK, Glueck D, Tench K. No effect of menstrual cycle phase on glucose kinetics and fuel oxidation during moderate-intensity exercise. American Journal of Physiology-Endocrinology and Metabolism 2002; 282(4):E752–E762.

68. Frandsen J, Pistoljevic N, Quesada JP, et al. Menstrual cycle phase does not affect whole body peak fat oxidation rate during a graded exercise test. J Appl Physiol 2020; 128(3):681–687.

69. Tanabe K, Kamemoto K, Kawaguchi Y, et al. Publisher Correction: Proteomics of appetite-regulating system influenced by menstrual cycle and intensive exercise in female athletes: a pilot study. Sci Rep 2024; 14(1):6798.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Chapter 7. Conclusions of the International Doctoral Thesis and future perspectives

1. Conclusions

- The time-of-day of a single bout of exercise does not influence the systemic effects on blood pressure, glucose regulation, energy metabolism and perceived appetite feelings in healthy men and women (Specific aim 1, 2, and 4).
- There exists sexual-dimorphism and time-of-day variations in the transcriptome, mitochondrial proteome and mitochondrial function of the skeletal muscle in response to moderate-intensity aerobic exercise, although one single session is not enough to reflect it in systemic adaptations (Specific aim 3).
- The time-of-day of moderate-intensity aerobic exercise impact more importantly on women's skeletal muscle, resulting exercise a stronger stimulus in the evening and producing a metabolic response with higher reliance on carbohydrates, increased cytokine expression and increased potential to suppress appetite in the short-term (Specific aim 3 and 4).
- Women present significant expression of the enzyme CNDP2 in the skeletal muscle only after evening moderate-intensity aerobic exercise and exhibit a differential pattern in the exercise-induced release of Lac-Phe. Lac-Phe, lactate, and IL-6 are linked to appetite feelings only in the evening in women (Specific aim 4).
- Adaptations in men's skeletal muscle display inhibited carbohydrate utilization and possibly enhanced mitochondrial function and fat utilization after evening moderate-intensity aerobic exercise, although time-of-day variations are less evident than in women (Specific aim 3).
- Regardless of the time-of-day, moderate-intensity aerobic exercise has minimal impact on acute appetite regulation in men (Specific aim 4).

While these results are not sufficient to create clinical recommendations, the present International Doctoral Thesis provides a molecular framework for future research. By identifying and understanding the molecular underpinnings of exercise responses, exercise professionals can make more targeted, personalized prescriptions, potentially optimizing therapeutic benefits.

2. Future perspectives

The findings from this International Doctoral Thesis underscore the importance of considering sex-specific differences when designing exercise interventions. There is a need to precisely define the diurnal variation in the physiological and molecular effects of exercise between men and women, taking into account the type of exercise, the specific hour within morning and evening, and health status.

Intensity plays a crucial role in the time-of-day variation of the metabolic response to exercise. A higher intensity of exercise in men compared to women may be necessary to observe time-of-day dependent adaptations to exercise.

The specific hour within the morning and afternoon/evening can influence the response to exercise at different times of the day. Women have a shorter circadian period (i.e., earlier phase shift), what suggests that not all exercise hours, particularly in the evening, are comparable between sexes.

Health status appears to affect the time-of-day impact of exercise on cardiometabolic parameters. While type 2 diabetes mellitus has been broader studied in this context, other conditions like metabolic syndrome or obesity remain providing conflicting results. Conflicts in this topic could be partially addressed by investigating how meal timing relative to morning and evening exercise (i.e., meal after exercise) interacts with cardiometabolic outcomes.

ANNEX: SHORT CURRICULUM VITAE

Short Curriculum Vitae

Summary

Raquel Sevilla Lorente (RSL) holds a bachelor's degree in Human Nutrition and Dietetics from the University of Granada (2019), partially completed at "Via University College" in Aarhus, Denmark, through a five-month Erasmus scholarship (2018). RSL graduated with an honorary mention for exceeding the 90th percentile in her promotion and received the International Excellence Award from the Faculty of Pharmacy for achieving the highest score in her international internship. During her bachelor's degree, she received a Collaboration Fellow from the Spanish Ministry of Education, which allowed her to begin research in the Department of Biochemistry and Molecular Biology II. She completed a master's degree in Molecular Biology Applied to Biotechnology Companies (2020), and before finishing, she was awarded the prestigious pre-doctoral grant in Spain (FPU19/03745 grant, 2019-2024) to pursue her PhD thesis.

As a doctoral candidate, she has led the DIVA study as project manager, which encompasses a team of more than 30 members across two research centers in the University of Granada (Mixed University Institute of Sport and Health, and Biomedical Research Center) and one Hospital (San Cecilio University Hospital, Granada). The overall aim of the DIVA study was to investigate the diurnal variation of moderate-intensity aerobic exercise on glucose regulation and energy metabolism in healthy men and women, and to elucidate the underlying molecular mechanisms in the skeletal muscle (ClinicalTrials.gov ID: NCT05369715). Recently, the DIVA study has been awarded with the second prize in National Sports Medicine Research Awards (Cajatsur Foundation, 2024). RSL also completed a research stay at the University of Clermont-Auvergne (Clermont-Ferrand, France) under the mentorship of Prof. David Thivel (September-December 2022). This stay was supported by the complementary mobility scholarship for FPU program beneficiaries. At the University of Clermont-Auvergne she received training in the study of appetite feelings and food reward in response to exercise. After this stay, RSL received €1,000 in funding from the University of Granada: "Projects for PhD Students" (2023), to validate the Spanish version of the most accepted tool for measuring food reward, the "Leeds Food Preference Questionnaire". RSL also undertook a research stay at the Queensland University of Technology (Brisbane, Australia) from April to August 2023, under the guidance of Prof. Niel King. This research stay provided her with further training to examine the diurnal variation of the effect of exercise on energy metabolism and particularly appetite related outcomes.

RSL has contributed to 4 research articles, with 1 as the first and corresponding author (50% appearing in Q1 and 50% appearing in Q2). She has also submitted 9 meeting abstracts, with 4 as the first author. Further, she maintains an active presence in scientific dissemination, and she has been invited to participate as a content creator or speaker in several public events.

EDUCATION

2020-2024 PhD Student in Biomedicine, University of Granada, Spain.

2019-2020 Master's degree in Molecular Biology Applied to Biotechnology Companies (Grade: 9.1/10) Faculty of Pharmacy, University of Granada, Spain.

2013-2017 Bachelor's degree in Human Nutrition and Dietetics [(Grade: 8.8/10), Summa Cum Laude in: Structural Biochemistry, Metabolic Biochemistry, Human Physiology, Community Nutrition, Advanced Bromatology, Food Hygiene and Safety, Food Parasitology, and Nutrition in Physical Activity and Sport], Faculty of Pharmacy, University of Granada, Spain.

NATIONAL FELLOWSHIPS

2020-2024 FPU pre-doctoral research fellowship (FPU19/03745) for PhD studies. Funded by the Spanish Ministry of Universities: 56,000€.

2018 Collaboration fellowship. Funded by the Spanish Ministry of Universities: 2.000,00€

INTERNATIONAL INTERNSHIPS

2022 Metabolic Adaptations to Exercise under Physio-pathological conditions Research Group, Université Clermont-Auvergne, Clermont-Ferrand, France. Duration: 3 months. Funded by the Spanish Ministry of Universities: 4200€.

2018 Via University College, Aarhus, Denmark. Duration: 5 months. Funded by the European Union (Erasmus+): 1500€.

PUBLICATIONS

Sevilla-Lorente R, Carneiro-Barrera A, Molina-Garcia P, Ruiz JR, Amaro-Gahete FJ. Time of the day of exercise impact on cardiovascular disease risk factors in adults: a systematic review and meta-analysis. J Sci Med Sport. 2023. Q1. IF: 3.0. SPORT SCIENCES, RANK: 22/127.

Ruiz JR, **Sevilla-Lorente R**, Amaro-Gahete FJ. Time for precision exercise prescription: the same timing may not fit all. J Physiol. 2023. Q2. IF: 3.1. PHYSIOLOGY, RANK: 22/127.

Camacho-Cardenosa A, Clavero-Jimeno A, Martin-Olmedo JJ, Amaro-Gahete F, Cupeiro R, Cejudo MTG, García Pérez PV, Hernández-Martínez C, **Sevilla-Lorente R**, et al. Time-restricted eating and supervised exercise for improving hepatic steatosis and cardiometabolic health in adults with obesity: protocol for the TEMPUS randomised controlled trial. BMJ Open. 2024. Q2. IF: 2.4. MEDICINE, GENERAL & INTERNAL, RANK: 80/325.

Molina NM, Jurado-Fasoli L, Sola-Leyva A, **Sevilla-Lorente R**, Canha-Gouveia A, Ruiz-Durán S, Fontes J, Aguilera CM, Altmäe S. Endometrial whole metabolome profile at the receptive phase: influence of Mediterranean Diet and infertility. Front Endocrinol (Lausanne). 2023. Q1. IF: 3.1. ENDOCRINOLOGY & METABOLISM, RANK: 51/186.

CONFERENCE ABSTRACTS

Only the conference abstracts as first author are included:

Sevilla-Lorente R, Marmol-Perez A, Gonzalez-Garcia P, Rodríguez-Miranda N, Riquelme-Gallego B, Aragon-Vela J, Martinez-Gálvez JM, Molina-Garcia P, Alcantara JMA, Garcia-Consuegra J, Cogliati S, Salmeron LM, Huertas JR, Lopez LC, Ruiz JR*, Amaro-Gahete FJ*. *Equally contribution. Cell Symposium: Exercise Metabolism. Cell Symposia. 2024. Portugal.

Sevilla-Lorente R, Mármol-Pérez A, Rodríguez-Miranda N, Riquelme-Gallego B, González-García P, Martínez-Gálvez JM, López LC, Huertas JR, Ruiz JR, Amaro-Gahete FJ. Efecto de la hora del ejercicio sobre las sensaciones de apetito, insulina y glucosa en sangre en hombres y mujeres con normopeso. VIII Simposio EXERNET: Ejercicio físico para la salud a lo largo de la vida. Red Española de Investigación en Ejercicio Físico y Salud. 2023. España.

Sevilla-Lorente R, Carneiro-Barrera A, Molina-Garcia P, Ruiz JR, Amaro-Gahete FJ. Efecto de la hora del día del ejercicio sobre los factores de riesgo cardiovascular: una revisión sistemática y meta-análisis. II Congreso Investigación PTS. Fundación PTS. 2022. España.

Sevilla-Lorente R, Marmol-Perez A, Medrano M, et al, Amaro-Gahete FJ. Variación diurna del efecto del ejercicio aeróbico sobre el metabolismo de la glucemia y la oxidación de grasas en humanos: rol del sexo y el peso corporal. II Congreso Investigación PTS. Fundación PTS. 2022. España.

PARTICIPATION IN RESEARCH PROJECTS

DIVA Project: “Diurnal Variation of Exercise on Metabolic Health”. PI: Jonatan Ruiz Ruiz. Funding: Scientific Excellence Unit of Exercise, Nutrition, and Health. Project Manager.

LFPQ-SP Project: Psychometric properties and validity of the cultural adaptation of the Spanish version of the 'Leeds Food Preference Questionnaire'. Funding: Research and Transfer Plan of the University of Granada (Program P.20.b., 2022) and Scientific Excellence Unit of Exercise, Nutrition, and Health (UCEENS). Project Manager.

TEMPUS Project: Time-restricted eating and supervised exercise for improving hepatic steatosis and cardiometabolic health in adults with obesity. PI: Jonatan Ruiz Ruiz. Funding: Spanish Ministry of Science, Innovation and Universities (PID2022-141506OB-I00, the European Regional Development Funds (ERDF), Agencia Estatal de Investigación, and Scientific Excellence Unit of Exercise, Nutrition, and Health (UCEENS). Research Team Member.

METANUT Project: Female reproductive health and uterine metabolome: influence of nutrition. PI: Nerea Molina Morales. Funding: Research and Transfer Plan of the University of Granada (Program P.20.b., 2021). Research Team Member.

EXTREME Project (pilot study): Adherence, safety, tolerability, and general feasibility of time-restricted eating in overweight/obese adults. PI: Jonatan Ruiz Ruiz. Funding: Scientific Excellence Unit of Exercise, Nutrition, and Health. Research Team Member.

UNIVERSITY TEACHING

2021-2024 Human and Cellular Physiology (146 hours of teaching, 14.6 ECTS [European Credit Transfer and Accumulation System]). Degree/Bachelor: Pharmacy, Human Nutrition and Dietetics and Physical Activity and Sport Sciences, University of Granada (Spain).

2021-2023 Physiopathology (25 hours of teaching, 2.5 ECTS [European Credit Transfer and Accumulation System]). Degree/Bachelor: Pharmacy and Human Nutrition and Dietetics, University of Granada (Spain).

2021 Clinical Physiology and Biochemistry (2.5 hours of teaching, 0.25 ECTS [European Credit Transfer and Accumulation System]). Degree/Bachelor: Pharmacy, University of Granada (Spain).

2021 Molecular Physiology of Animals (6.5 hours of teaching, 0.65 ECTS [European Credit Transfer and Accumulation System]). Degree/Bachelor: Biochemistry, University of Granada (Spain).

PRIZES AND AWARDS

2024 Second prize in National Sports Medicine Research Awards for the DIVA Project, Cajatsur Foundation, University of Oviedo (Spain).

2023 The paper "Time of the day of exercise impact on cardiovascular disease risk factors in adults: a systematic review and meta-analysis" was top 3 highlighted by the Editors of Journal of Science and Medicine in Sport in March 2023.

2019 Honorary mention from the Faculty of Pharmacy for exceeding the 90th percentile during bachelor's degree in Human Nutrition and Dietetics, University of Granada (Spain).

2019 International Excellence Award from the Faculty of Pharmacy, University of Granada (Spain).

DISEMINATION AND INVITED SPEAKER

2023 Desgranando Ciencia. Asociación de divulgación científica con el objetivo de acercar la ciencia y el pensamiento crítico a la sociedad. Teatro Isabel la Católica, Granada (Spain).

2021 III Jornadas “No todo es ciencia” de divulgación científica, pensamiento crítico y creativo. Universidad de Pablo Olavide, Sevilla (Spain).

2021 Health Campaing against Eating Disorders "No Seas Presa de la Talla", 2021, Federación de Mujeres Jóvenes Funded by the Ministry of Social Rights and Agenda 2030. Content creator: illustrations. https://mujeresjovenes.org/wp-content/uploads/2024/03/Guia-de-recursos_No-seas-presa-de-la-talla.pdf

LANGUAGES

Certificate in Advanced English (CAE) by Cambridge. Council of Europe level C1.