ROLE OF EXERCISE, COLD, AND THE BIOLOGICAL CLOCK ON ENERGY BALANCE IN HUMANS: THE ACTIBATE STUDY

DOCTORAL PROGRAMME IN BIOMEDICINE

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UNIVERSIDAD DE GRANADA

Role of exercise, cold, and the biological clock on energy balance in humans

The ACTIBATE study

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Role of exercise, cold, and the biological clock on energy balance in humans: The ACTIBATE study

Efecto del ejercicio físico, frío y factores relacionados con el ritmo circadiano sobre el balance energético en humanos

PROGRAMA DE DOCTORADO EN BIOMEDICINA

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Francisco M. Acosta

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A mis padres, hermanos y amigos cercanos. En especial, a aquellas personas que me ayudaron a descubrir la belleza de la ciencia

To my parents, siblings, and close friends. Specially, to those who help me discovering the beauty of science

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RESEARCH PROJECTS AND FUNDING

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ABSTRACT/RESUMEN

ABSTRACT

Cold exposure has been proposed as a potential tool to face obesity since it is able to increase energy expenditure (i.e., cold induced thermogenesis – CIT). This increase is partially mediated by brown adipose tissue (BAT). Nevertheless, little is known yet about how CIT (specially its non-shivering component) and BAT metabolic activity are regulated, and which are the clinical implications of harnessing them in humans. Furthermore, most physiological functions in our organism are under the control of the biological clock, which shows a tight link with disease in humans. However, to date it remains unknown whether CIT and BAT activity follow a diurnal/circadian rhythmicity, and whether they are related to the general functioning of the circadian system. Furthermore, whether exercise and physical activity are efficient strategies to recruit and activate BAT still need to be explored. Therefore, the overall aim of the present Doctoral Thesis was to study the role of cold and the biological clock on energy balance in humans as well as on BAT; and to investigate whether exercise and physical activity could be potential strategies to recruit and activate BAT in humans.

Mild cold elicits a modest increase in CIT and prompts a fat oxidative metabolism, especially during the initial moments of cold exposure **(Study I).** In addition, subjects exposed to the coldest ambient temperature during their daily life have 3-5 times more BAT volume and activity - measured as ^{18}F-fluordeoxygelucose (^{18}F-FDG) uptake - compared to subjects who are exposed to a warmer ambient temperature **(Study II).** CIT and BAT ^{18}F-FDG uptake do not seem to have diurnal variations **(Study III),** and the relationship between the daily rhythm of distal skin temperature - an overall indicator of the circadian system functioning - and BAT ^{18}F-FDG uptake, is masked by the environmental temperature **(Study II)**. In addition, sleep duration and quality are not related to human BAT metabolic activity **(Study IV).** An acute aerobic exercise does not induce significant changes in the protein concentrations of UCP-1 (and therefore BAT activity) in mice **(Study VI)**. Accordingly, physical activity levels and sedentary time are not related to BAT ^{18}F-FDG uptake in young human adults **(Study VII)**, neither to the regulation of browning markers, suggesting a negligible role for exercise and physical activity as potential strategies to recruit and activate human BAT.

The results from the present Doctoral Thesis increase our knowledge on the effects of cold on energy and BAT metabolism in humans, and on how the biological clock and related factors might affect them. In addition, it provides novel information on the search of potential strategies to improve BAT function and browning of specific adipose depots.

RESUMEN

La exposición a frío puede tener un gran potencial para combatir la obesidad, a través del incremento de la termogénesis inducida por frío (CIT*, del inglés*), la cual está mediada parcialmente por el tejido adiposo pardo (TAP). Sin embargo, hay poca evidencia científica en torno a cómo el CIT (y más concretamente su componente sin tiritona) y la actividad del BAT son regulados – así como cuales son las implicaciones incrementar ambos en humanos. Además, la mayor parte de las funciones fisiológicas de nuestro organismo están bajo el control del reloj biológico, que está estrechamente vinculado con la salud en humanos. Sin embargo, se desconoce si el CIT y el TAP presentan una ritmicidad diurna/circadiana. Si el ejercicio y la actividad física son estrategias eficientes para reclutar y activar el TAP también necesita ser estudiado en mayor profundidad. Por lo tanto, el objetivo general de la presente tesis doctoral es estudiar el rol del frío y el reloj biológico en el balance energético en humanos, así como en el TAP; e investigar si el ejercicio y la actividad física podrían ser estrategias potenciales para reclutar y activar el TAP.

La exposición a frío moderado induce un incremento ligero en el CIT y promueve una mayor oxidación de grasas, especialmente durante el comienzo de la exposición a frío **(Estudio I).** Además, los participantes que se expusieron a temperaturas más frías durante su vida diaria tenían de 3 a 5 veces más volumen y actividad del TAP – medido como captación de ^{18}F-Fluordeoxiglucosa (^{18}F-FDG) – en comparación con aquellos que se expusieron a temperaturas más cálidas **(Estudio II).** El CIT y la captación de ^{18}F-FDG por el BAT no parecen seguir variaciones diurnas **(Estudio III),** y la relación entre el patrón diario de la temperatura superficial distal – utilizada como un indicador general del funcionamiento del sistema circadiano – y la captación de ^{18}F-FDG del TAP, está enmascarada por la temperatura ambiental (**Estudio II).** La duración y calidad del sueño tampoco parecen relacionarse con la actividad metabólica del TAP en humanos **(Estudio IV).** Además, encontramos que el ejercicio aeróbico agudo no induce cambios significativos en los niveles de la proteína UCP1 (y por lo tanto en la actividad del TAP) en ratones **(Estudio VI).** En línea con esto, los niveles de actividad física y el tiempo en sedentarismo no se relacionan con la captación de ^{18}F-FDG del TAP en jóvenes adultos humanos (Estudio VII), ni a la regulación de marcadores de “amarronamiento”; esto

sugiere que el ejercicio y la actividad física tienen un rol insignificante como estrategias potenciales para reclutar y activar el TAP.

Los resultados de la presente tesis Doctoral extienden nuestro conocimiento sobre los efectos del frío sobre el metabolismo energético y del TAP en humanos, y sobre como el reloj biológico y factores relacionados al mismo podrían afectarles. Además, proporciona información valiosa sobre la búsqueda de estrategias para mejorar la función del BAT, e inducir el "amarronamiento" de depósitos específicos de tejido adiposo.

ABBREVIATIONS

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^{18}F-FDG: ^{18}F-fluordeoxyglucose

ACTIBATE: activating brown adipose tissue through exercise

AMPK: adenosine monophosphate-activated protein kinase

ANCOVA: analyses of covariance

ANOVA: analysis of variance

ATF2: activating transcription factor 2

ATP: adenosine triphosphate

AUC: area under the curve

AVP: vasopressin (or arginine vasopressin)

BAT: brown adipose tissue

BCA: bicinchoninic acid

BMAL1: brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT) like 1

BMI: body mass index

BMR: basal metabolic rate

BSR: burst shivering rate

CHO_{ox}: carbohydrates oxidation rate

CI: cold induced

CIT: cold-induced thermogenesis

CLOCK: circadian locomotor output cycles Kaput

CP: cold period

CRY1: cryptochrome circadian regulator 1

CRY2: cryptochrome circadian regulator 2

CT: computed tomography

CV: coefficient of variance

Dio2: type 2 iodothyronine deiodinase

DIT: diet induced thermogenesis

DLMO: dim light melatonin

DMN: dorsomedial nucleus

DST: distal skin temperature

DXA: dual-x energy ray absorptiometry

Abbreviations

EDTA: ethylenediaminetetraacetic acid

EE: energy expenditure

EMG: electromyography

ENMO: euclidean norm minus one

eWAT: epididymial white adipose tissue

FAT_{ox}: fat oxidation rate

FGF21: fibroblast growth factor 21

FMI: fat mass index

FTHA: fluoro-6-thia-heptadecanoic acid

GABA: gamma-aminobutyric

GLUT4: glucose transporter 4

HFD: high fat diet

HHb: deoxyhaemoglobin

HOMA: homeostatic model assessment of insulin resistance

HU: hounsfield units

iBAT: interscapular brown adipose tissue

IL-6: interleukin 6

IL-6Rα: interleukin-6 receptor alpha

ILM: intermedio-lateral nucleus

IS: interday stability

IV: intraday variability

JAK: janus kinase

kBq: kilobecquerel

kV: kilovoltios

L10: ten consecutive hours with the minimum DST values

LDL-C: low density lipoprotein - cholesterol

LMI: lean mass index

LPA: light physical activity

M5: five consecutive hours with the maximum DST values

mA: miliamperios

MARP: Monash animal research platform

MBq: megabecquerel

MIPS: Monash Institute of Pharmaceutical Science

MIT: meal induced thermogenesis

ml: millilitre

MnPO: median preoptic subnucleus

MPA: moderate physical activity

mRNA: messenger ribonucleid acid

MVPA: moderate-vigorous physical activity

$MVPA_{10min}$: moderate-vigorous physical activity in bouts of 10 consecutive minutes

NEAT: non-exercise activity thermogenesis

NIR_{SRS}: near infrared spatially resolved spectroscopy

$NIRS_{TRS}$: near infrared temporally resolved spectroscopy

NP-40: tergitol-type NP-40/ nonyl phenoxypolyethoxylethanol

NREM: non-rapid eye movement

NUT_{ox}: nutrients oxidation rate

O2Hb: oxyhemogloin

PA: physical activity

pCREB: phosphorylated CAMP responsive element binding

PER2: perlipin 2

Personal-ETL10: ten consecutive hours with the lowest values for the personal level of environmental temperature

PET/CT: positron emission tomography combined with computed tomography

PGC1α: coactivator-1-alpha of peroxisome proliferator-activated receptor gamma

PKA: protein kinase A

PMSF: phenylmethylsulfonyl fluoride

p-p38 MAPK: phosphorylated p38 monoadenosine phosphate kinase

PPARα: peroxisome proliferator-activated receptor alpha

PPARγ: peroxisome proliferator-activated receptor gamma

PRO_{ox}: protein oxidation rate

PSQI: Pittsburgh Sleep Quality Index

pSTAT3: phosphorylated STAT3

REE: resting energy expenditure

REM: rapid eye movement

Abbreviations

RGC: retinal ganglion cells

RMS: root mean square

ROI: region of interest

RQ: respiratory quotient

rRPpa: rostral raphe pallidus

SCN: suprachiasmatic nucleus

sWAT: subcutaneous white adipose tissue

SDS: sodium dodecyl sulfate

SERCA: sarcoplasmic/endoplasmic reticulum calcium ATPase

SPSS: Statistical Package for the Social Sciences

ST: shivering threshold

STAT3: signal transducer and activator of transcription 3

STT: shivering threshold test

SUV: standardized uptake value

$SUVB_M$: standardized uptake value relativized to body mass

SUV_{LBM}: standardized uptake value relativized to the lean body mass percentage

SUV_{mean}: mean standardized uptake value

SUV_{peak}: peak standardized uptake value

TBST: tris-buffered saline + polysorbate 20

TBX1: T-Box Transcription Factor 1

TEE: total energy expenditure

TEF: thermic effect of food

TGF: tumour growth factor

tHb: total haemoglobin

TL10: time at which L10 happens

TM5: time at which M5 happens

TMEM26: transmembrane Protein 26

TSI: tissue saturation index

UCP1: uncoupling protein 1

USA: United States of America

VAS: visual analogue scales

VAT: visceral adipose tissue

VCO_2: volume of carbon dyoxide production

VIP: vasoactive instestinal polypeptide

VO_2: volume of oxygen consumption

VO_2 max.: maximum volume of oxygen consumption

VPA: vigorous physical activity

WASO: wake after sleep onset

WAT: white adipose tissue

WP: warm period

β3AR: B3-adrenergic receptor

GENERAL INTRODUCTION

GENERAL INTRODUCTION

OBESITY: A PANDEMICS WITHOUT SOLUTION?

Overweight and obesity prevalence is globally increasing, and data indicate that approximately 1.30 billion adults are overweight and 335 million are obese[1]. These figures are worrisome, and identify obesity as a pandemic which is far from being resolved. One of the problematics of obesity, is that it is tightly linked to a wide array of comorbidities and diseases. Obesity is related to insulin resistance, type 2 diabetes, hypertension, dyslipidaemia, coronary heart disease, certain tumours, and to a more pro-inflammatory profile, among others [2,3]. This situation represents a general concern not only for people, but also for the health care systems, which are unable to face the increasing financial burden associated with obesity. Considering all above, effective strategies are urgently needed.

Despite the fact that obesity is determined by genetic, environmental, behavioural, and social factors[4], a simplistic definition has been traditionally used to approach it: "obesity is driven by energy imbalance favouring storage of the energy surplus not expended, or in other words, it is the result of a daily energy intake exceeding total energy expenditure (TEE)"[4]. TEE is a multicomponent construct **(see Figure 1),** explaining resting energy expenditure (REE) the major part of it (approximately 60-70%), together with other components such as physical activity related energy expenditure and adaptive thermogenesis (meal and cold-induced thermogenesis)[5–7].

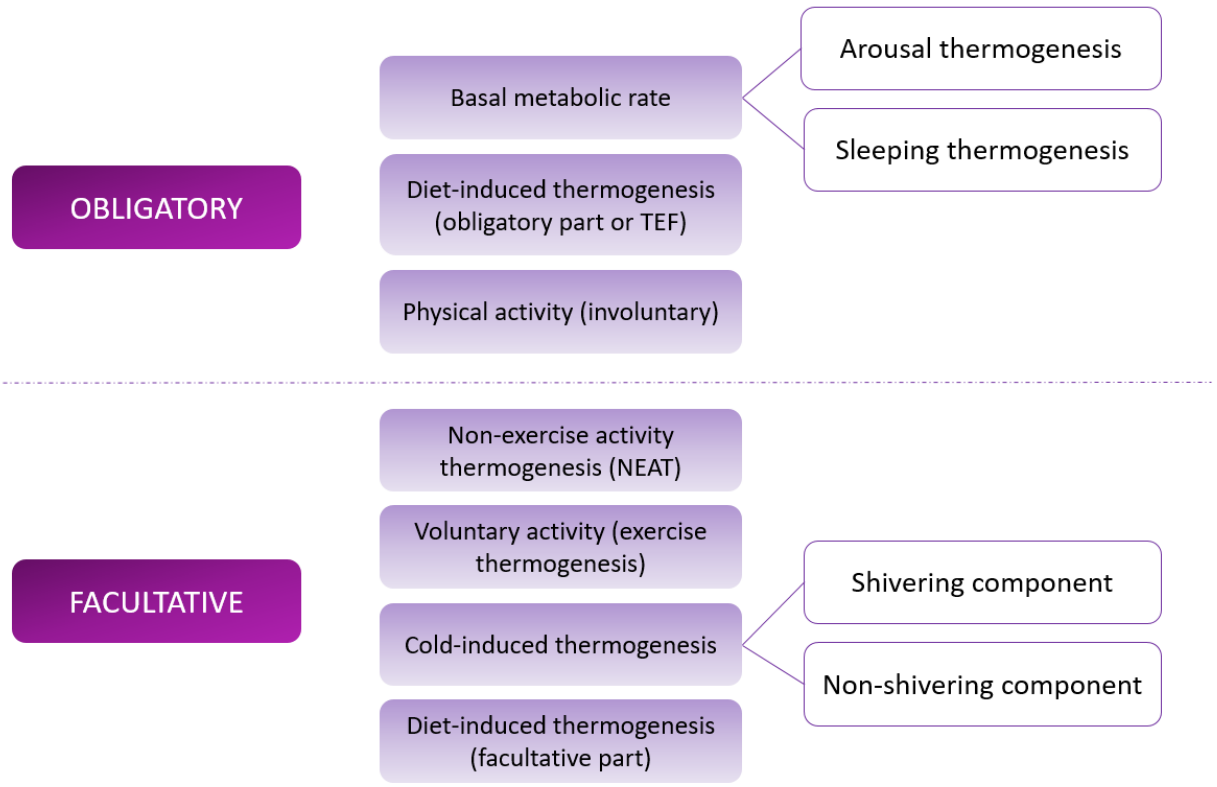

Figure 1. Component of energy expenditure in humans (not considering additional phenomenon such as growing or reproduction). Adapted from Van Marken et al.[5] TEF: thermic effect of food.

Theoretically, and if not compensated by any compensatory mechanism, reduction in food intake, or increases in any of previous components could lead to changes in body weight, and therefore become potential strategies to face obesity and related comorbidities. Nevertheless, this scenario is quite more complex than it might initially look.

Firstly, we live in an obesogenic environment, where obesity behaviours are promoted by our social interactions and built environment[4]. This is reflected by the consumption of junk and processed food, high fat and sweet products, the use of private transport in detrainment of active transport, the large amount of time spent in sedentary behaviours, such as watching tv or playing with videogames, and the use of constant indoor temperatures within our thermal comfort range, among others. This obesogenic environment, together with the lack of adherence at long-term (due to psychobiological factors), or responsiveness to the most implemented weight-loss interventions – caloric restriction and physical exercise - greatly difficult the implementation of effective strategies to face obesity.

Furthermore, due to evolutionary purposes (e.g., survival to famine, hunting during long fasting periods, etc.), our genetic code seems to be prepared to function as an energy saver (i.e., "thrifty theory)[4]. Accordingly, a myriad of compensatory mechanisms, far from been understood, take place when we are submitted to a negative energy balance at long term[4,8]. For instance, when caloric restriction plans are carried out, an initial and pronounced weight loss can be observed (e.g., up to 50.6 kg or 40% initial body mass in 30 weeks, in severely obese subjects)[9]. Nevertheless, only a small percentage – 15% of those participants who are involved in a diet-weight loss program have long-term success in weight maintenance[10]. This is explained by weight-loss induced reductions in resting and non-resting EE[11], which is partially driven by the loss of skeletal muscle mass[12]. However, it has been shown that even when the loss of skeletal muscle mass is relatively minimized with an exercise program[8], a decrease in the non-resting EE can still be observed, which seems to be explained by an increase in the metabolic efficiency of the contracting skeletal muscle, or decreased habitual PA levels[11]. Furthermore, diet and exercise interventions also provoke changes in the regulation of different hormones and cytokines related to the regulation of appetite, which may lead to increased energy intake[13,14]. Therefore, little is known about how to efficiently face obesity. Indeed, we are far from understanding the compensatory mechanisms happening in programs which target weight-loss.

Since strategies aimed at decreasing energy intake (alone or in combination with exercise) are generally ineffective, one solution may be to tackle the other branch of energy balance, such as increasing REE, PA related energy expenditure, or adaptive thermogenesis. As previously mentioned, REE is the main contributor to TEE, and is mainly dependent on the skeletal muscle mass. Despite the fact that REE is modifiable through resistance exercise programs (aiming to increase the skeletal muscle mass), it may be a component difficult to modify since it requires a large adherence and periodization of the stimulus. Regarding the increase of PA related energy expenditure, Pontzer et al.[15] recently proposed a model in which EE is increased until PA levels surpass a certain threshold. Based on this model, known as the "Constrained EE", they proposed that the relationship between PA levels and NEAT (non-exercise activity thermogenesis) is linear until a specific threshold is reached. From there, beyond, a plateau in NEAT can be observed, which is not attributable to a reduced REE, but to

changes in the EE related to reproductive and somatic activities. Hence, and since the previous components are less susceptible to modification or seem to have a roof effect, the search for new/ complementary strategies is necessary.

Among these strategies, one of the most promising is to increase adaptive thermogenesis, which is composed by both, diet and cold-induced thermogenesis (DIT and CIT, respectively). DIT is composed by an obligatory component - which refers to the energy spent for digestion, absorption, processing and storage of nutrients after a meal -, and a facultative component - related to the heat produced after a meal[16]. DIT divergently contributes to TEE based on the macronutrient intake of diet, and it has been proposed that it can account for nearly a 5-15% of daily TEE in individuals consuming a mixed diet [7,16]. DIT is increased mainly through diets with high protein content (which is related to the ATP requirements to convert nutrients to metabolic fuel), but how to exploit DIT requires further research. The other component of adaptive thermogenesis is CIT, a physiological response that takes place when our body is not able to fully compensate for heat loss by changes in skin blood flow or our behaviour (e.g., clothing, use of central heating, etc.)[17], increasing energy expenditure in response to a cold exposure. In comparison with MIT; this physiological response is more easily modifiable, and can increase TEE to a much larger extent[18]. Importantly, the environmental temperature to which we are exposed every day can be modified thanks to the advancement in technologies, which allow us to switch on/off air-conditioning or heating by just pressing a bottom. Hence, CIT has been proposed as a promising strategy to create a negative energy balance and face obesity and its related comorbidities[19].

COLD AS A THERAPEUTIC STIMULUS: PHYSIOLOGICAL MECHANISMS AND CLINICAL IMPLICATIONS IN OBESITY AND RELATED COMORBIDITIES

Humans are endothermic beings, which means that they are able to modulate their heat production to compensate for heat dissipation, maintaining a stable core temperature (i.e., set point) against environmental challenges[17,20]. In cold temperatures, and when changes in the behaviour and skin blood flow are not able to fully compensate heat loss, energy expenditure increases, an adaptive physiological response known as CIT[6,17]. It has been shown that in small mammals (i.e., mice), CIT

can increase energy expenditure up to 60% above REE under standard animal housing temperatures (~22-23ºC), and that when exposed at an ambient temperature of 4ºC, energy expenditure can be increased up to 4-5 times[21,22]. This clearly shows the substantial energetic demands of thermoregulatory effectors.

In humans, CIT response has been broadly studied using different methods (cold air, water immersions, water perfused garments, ice-blocks, among others), as well as different exposure times[6]. CIT is typically divided into 2 main components: shivering thermogenesis and non-shivering thermogenesis. Shivering thermogenesis increases heat production in response to cold mainly through the skeletal muscle contractile activity. It is able to provoke a 5-fold increase in REE in humans when shivering is maximum, being fundamental to avoid hypothermia and ensure human survival under extreme cold conditions[18,23,24]. However, shivering thermogenesis is often uncomfortable and fatiguing, and compromises locomotion. On the other hand, non-shivering thermogenesis is triggered mainly by mild cold and normally increase REE in a range from 15 to 30% above REE, without inducing a large discomfort[25]. Consequently, increasing CIT, and specially, its non-shivering component, have become attractive targets as health-promoting stimulus to counteract obesity and related comorbidities[8,26]. Nevertheless, humans present a much lower area surface to volume ratio than small mammals, and have a better capacity to regulate their behaviour, therefore, relying less on CIT to maintain a stable core temperature[17]. In addition, human CIT seems to present a high-inter-individual variability[6,27]. Hence, it is still unknown how we can safely and comfortably harness CIT in humans in the fight against obesity[6,28].

Importantly, the cold effect over human thermoregulation and metabolism has been studied for many years by several authors (Voit, 1878; Swift, 1932; Hardy & DuBois, 1937; Winslow et al., 1937; Brychta & Chen, 2017)[6,29–31], but specially during the last decades. Nowadays, it is well known that cold exposure is able to trigger a large range of physiological responses and adaptations in humans[32,33], integrating a multi-organ/systems response. Nevertheless, researchers have mainly focused on characterizing the metabolic effects of severe cold exposure, especially in shivering thermogenesis[34], although litlle is known yet about how non-shivering thermogenesis is regulated, and which are the mechanims and tissues/organs involved in this

physiological response[35]. Among other issues, it specially remains to be ascertained: i) the clinical implications of cold exposure under well periodized and lab-controlled conditions, as well as during free living conditions, on human health; ii) the role of BAT, a thermogenic organ recently "rediscovered" to be active in humans, which seems to mediate some of the physiological responses and benefits that cold exposure exerts in humans.

BROWN ADIPOSE TISSUE: FROM ITS REDISCOVERY TO ITS BIOLOGICAL SIGNIFICANCE

From a traditional perspective, the main functions of white adipose tissue have been related to its capacity as a nutrient reservoir (i.e., lipid storage), and as a shield against the exterior world. As time has gone by, more and more research has evidenced that the role of white adipose tissue is further more complex, and has a major endocrine function secreting several hormones that regulate the metabolic homeostasis and inflammatory profile[36]. A clear example is the discovery of leptin[37]. Since the discovery that it is mainly secreted by white adipose tissue, and it is an important mediator on obesity, cardiometabolic diseases, and inflammation[37] much research has focused on the metabolic role of white adipose tissue and its potential implications. At the same time, it has been recognized that adipose tissue is very heterogeneous and plastic, and different subtypes have been identified and further explored[38–42].

Among them, BAT - a tissue which was initially believed to be only present in neonates and small mammals[43] - has gained a plethora of attention during the last decade. This is explained because in 2007, Barbara Cannon and Jan Nedergaard, found that BAT was present and metabolically active in adult humans[44]. This fact was confirmed by the subsequent publication of 5 different studies in 2009[45–49], confirming that functional BAT exists in adult humans, in both thermoneutral and cold conditions, leading to a change of paradigm. In addition, 2 of these studies confirmed the molecular signature of BAT in human biopsies taken from the neck area where BAT was meant to be localized, definitely confirming these findings[47,48]. Anecdotally, few previous reports had already noted existence of this tissue. In the 16th century, the Swiss naturalist Conrad Gessner reported that it was "neither fat, nor flesh [nec pinguitudo, nec caro], but something in between"[50]. Few centuries later -in 1972-, Heaton[51] dissected several human corpses and reported that interscapular BAT was

present, especially during the first decade of life, but that it progressively decreased with age. Posterior studies showed that those outdoor workers/people exposed to cold temperatures, or people living in cold regions, have a higher BAT prevalence than people exposed to warmer temperatures[52,53]. Similarly, Cohate at al.[54] found a significant ^{18}F-FDG uptake in in tissues of low-Hounsfied (fat) density, typical from BAT, that they called the USA-fat. Nevertheless, these studies (and others not mentioned)[50,55,56] passed unperceived until the rediscovery and confirmation of BAT, thanks to the former studies mentioned.

Brown adipose tissue is a thermogenic organ characterized by its capacity to dissipate energy in the form of heat through the canonical UCP1 protein[57]. UCP1 is an ATPase located in the inner membrane of the mitochondria, which allows the leakage of protons from the mitochondrial matrix to the intermembrane space. Therefore, UCP1 uncouples the energy substrate oxidation from ATP production (i.e., oxidative phosphorylation)[58]. Of note, BAT is a very heterogeneous tissue, formed by connective tissue, blood vessels, and brown adipocytes inter-spread with other adipocytes types, such as beige adipocytes. An intrinsic characteristic of brown adipocytes is that they are composed by numerous small lipid droplets (multiocular aspect), and have a high mitochondrial content. These characteristics normally provide to BAT its well differentiated brown colour (mainly due the high vascularization and abundant presence of cytochrome C).

BAT is mainly located in the supraclavicular, laterocervical, axillar, mediastinal, paravertebral and abdominal regions, as well as in the heart pericardium (see Figure 2)[59].

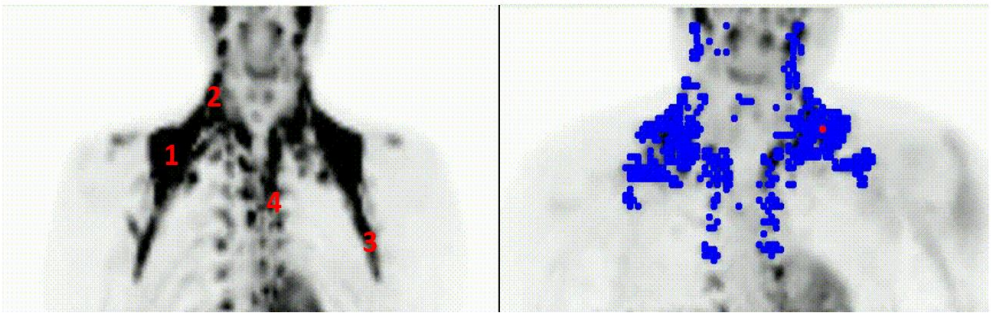

Figure 2. PET-CT scan of 2 young participants showing some of the typical areas where BAT is localized in humans. The image on the left shows BAT location based on the tissue radiodensity, whereas the image on the right shows BAT ^{18}F-FDG uptake, using the superposition of both, the CT and PET imaging components. Red numbers indicate BAT locations: 1, supraclavicular; 2, laterocervical; 3, axillar; and 4, mediastinal.

These depots are highly innervated by sympathetic projections, reflecting its canonical via of activation. In brief, thermal sensory receptors (e.g., TRMP8) located in the skin, viscera, spinal cord or brain are activated by specific stressors such as cold[17,60,61]. When these sensorial receptors (calcium channels) are activated, they transmit afferent signals to the primary sensory neurons located in the dorsal root ganglia, which are them projected into several nucleus/centres of the hypothalamus where they are integrated (e.g., median preoptic subnucleus –MnPO, and dorsomedial nucleus - DMN). Efferent signals are then released from these nuclei/areas to BAT sympathetic premotor neurons in the rostral raphe pallidus (rRPpa), which excite BAT sympathetic preganglionic neurons in the intermedio-lateral nucleus (ILM). Finally, these neurons project into BAT, releasing norepinephrine, the main neurotransmitter involved in BAT activation. Then, this ligand joins to B-adrenergic receptors in BAT membrane, and initiates a cascade through the G-protein and AMPK dependent signalling, that acts upregulating UCP1 expression. Of note, it still remains controversial which one is the main B-adrenergic receptor mediating this process. Recent studies have already discussed this issue[47,62,63].

Importantly, the biological importance of BAT, and its clinical implications, are a concern that still remain to be solved[50,64]. As previously mentioned, BAT has gained considerable attention as a potential therapeutic target for obesity and type 2 diabetes[6], based on evidence coming mainly from rodent models. Several studies have shown that BAT is inversely associated with body mass index and adiposity in humans[2–4]. Furthermore, it seems to have a role on the regulation of glucose and lipid metabolism[7,8] as well as to the improvement of peripheral insulin sensitivity[9]. In addition, those participants with high levels of BAT may be also those who present a decreased cardiovascular risk profile[50,65]. Nevertheless, evidence in humans is inconclusive and ambiguous, despite the inaccurate and the potential precipitated conclusions drawn by some authors. Further and more rigorous studies in humans are needed to advance on this field of research.

In line with all the hypothesised benefits of BAT on human health, the search of strategies to prompt BAT activation and recruitment is of great interest. Cold exposure is the main activator of BAT (together with B-adrenergic agonists, bile acids and adenosine), and it is generally safe and free of any cost. Until now, studies have

broadly confirmed that BAT is activated by cold under well controlled conditions[50]. Nevertheless, experimental conditions are often different from those to which participants are exposed during their daily life, when they can rely on behavioural responses (e.g., clothing, fidgeting, use of central heating, etc.) rather than physiological ones to face cold stress. Therefore, to understand how exposure to cold under free-living conditions affect to BAT needs to be addressed.

Of note, inter-spread within BAT, and other tissues such as WAT, it exists a subpopulation of cells which shares characteristics with brown and white adipocytes, the so-called beige or brite adipocytes[40,50]. Similar to brown adipocytes, these cells present UCP1, and are able to dissipate energy in the form of heat when stimulated by cold (although to a lower extent). They also present representative thermogenic markers such as TBX1 and TMEM26[39]. Beige adipocytes have a lower mitochondrial density, number of lipid droplets, vascularization, and innervation than brown adipocytes, but its thermogenic capacity have made them to be at the centre of attention. This is because white adipocytes can be converted into beige adipocyte – it is still unknown if by de novo differentiation, or transdifferentiation from white adipocytes - a process known as browning, which has been proposed as a future promising strategy against obesity[66,67].

THE FORGOTTEN ROLE OF THE BIOLOGICAL CLOCK ON HUMAN HEALTH: A FOCUS ON COLD-INDUCED THERMOGENESIS AND BROWN ADIPOSE TISSUE

The human body is a complex and living engine, which is the result of the constant interaction of the cells and components which form our organism. This engine, as the one of any machine, is composed by a set of gears that need to be perfectly greased and synchronized. If any of the components which form the whole, fail, the whole system will break. In other words, the correct working of our organism depends on the correct interaction of its components at all levels (cell, tissues, organs, systems), and a perfect timing/synchronization of them is necessary for a proper working of physiological functions. The desynchronization or misalignment of these functions will derive in homeostasis disruption and disease. This is an easy and graphic way to describe the importance of the biological clock in humans, but what is the biological clock, and how does it influence health?

Almost all living beings have a circadian time-keeping system[68] –so called biological clock. This circadian system consists of a network of hierarchically organized structures that regulate the body's temporal organization in relation to its environment[69–72]. In the case of humans, this entrainment is mainly produced based on the rotation of Earth around its axis, which imparts light and dark cycles of 24h. From an evolutionary perspective, this biological clock has allowed organisms to adapt their metabolic processes and their behaviour to cyclic environmental changes[71,73,74].

In mammals, the biological clock is composed by a central pacemaker, as well as by peripheral clocks[69,70,75,76] which are virtually existent in all tissues. The suprachiasmatic nucleus (SCN), also known as the master clock, is the central circadian oscillator, and is ventro-caudally located in the hypothalamus. This master clock is composed by a neuronal network, which is cell autonomous – i.e., it is composed by individual neurons able to define and express their own circadian rhythmicity[77]. When these individual oscillators are synchronized, they generate coordinated circadian outputs that regulate overt rhythms[78]. Circadian time in the SCN is mainly entrained by solar light. When the retina receives light input, a sub-population of retinal ganglion cells (RGC) in that area express melanopsin[79]. This protein contained in the axon of the sensory neurons, triggers an action potential which travels through the retinohypothalamic tract until the SCN core. In the SCN core, the gamma-aminobutiric acid (GABA) containing neurons, which express vasopressin (AVP) or vasoactive instestinal polypeptide (VIP) are activated. Then, these intrinsic factors to SCN, acutely activate and synchronize SCN neurons and coordinates behavioural rhythms[69,80].

Regarding the peripheral clocks or oscillators, they are present in most tissues. In fact, it has been shown that about 43% of protein-encoding genes in our body show circadian oscillations in their expression patterns, in an organ specific manner[81,82]. Despite these autonomous oscillations, peripheral clocks need to be orchestrated by the SCN to prevent their dampening. Hence, the SCN accomplish this task by sending neuronal connections (e.g., the SCN projects into the dorsal subparaventricular nucleus, which is implicated in the circadian rhythmicity of body temperature)[83], but also through the secretion of humoral factors (e.g., TGFα, prokineticin, cardiotrophin-like cytokine)[69]. At the same time, the SCN neurons can be influenced by neuronal and endocrine inputs[84].

Of note, the molecular mechanism underpinning the central pacemaker and peripheral clocks is the same[79]. This molecular machinery is based on a set of core-clock genes, forming a negative feedback loop. More specifically, the positive regulators of mammalian clock (transcription factors CLOCK and BMAL1) form a heterodimer in the nucleus of the cell, and induce the expression of clock-controlled genes by binding to their promoters at E-boxes. They regulate the expression of negative regulators of the mammalian clock – i.e., cryptochrome (CRY1 and CRY2) and period (PER1, PER2 and PER3). Once a critical concentration of these regulators is reached on the cytoplasm, they translocate into the nucleus and form a complex to inhibit the heterodimer CLOCK-BMAL1 mediated transcription, closing the negative feedback loop. The autoregulatory transcription-translation loop comprising CLOCK-BMAL 1 and PER-CRY constitutes the core clock and generate 24-h rhythms of gene expression. CLOCK-BMAL 1 heterodimer also prompts the transcription of Rev-erbα and RORα – which respectively stimulate and inhibit BMAL1 transcription.

As can be noted, the clock machinery is actually complex, and our knowledge about its relationship with human health/disease is still scarce[85]. Most evidence supporting this relationship comes from epidemiological studies[79]. For instance, it has been shown that social habits commonly disrupt the circadian system (chronodisruption) - as it occurs with shift workers-, leading to metabolic problems such as obesity, dyslipidemia or impaired glucose tolerance, to the development of certain tumours[86], and perhaps even shortening our life span[69–72]. Experimental evidence in healthy volunteers shows that circadian disruption induced by misalignments or sleep deprivation protocols have important implications on cardiometabolic health. For instance, it induces changes in the levels of fasting and postprandial glucose levels and in leptin and ghrelin concentrations, decreased peripheral insulin sensitivity, increase in the presence of inflammatory cytokines, etc.[87–89]. Certainly, the correct functioning of the circadian system seems necessary for properly and temporally adjust behavioural and physiological functions, and *vice versa*.

In line with this argument, it is tempting to speculate that circadian disruption in humans may be related to BAT dysfunction, which could have implications on health. Interestingly, in mice it has been shown that the formation and metabolic function of BAT are tightly related to the circadian clock regulation[73,90,91]. The BAT

transcriptome is robustly rhythmic[81,92], with nearly 8% of the tissue's expressed genes showing circadian rhythmicity. Accordingly, it has been shown that a number of key nuclear receptors involved in circadian rhythmicity, such as Rev-erb alpha and PER2, play essential roles in the modulation of murine UCP1 expression and BAT thermogenesis[90,93]. Studies in rodents have also shown that sleep regulation (including sleep duration, sleep-wake cycles, etc.) are closely related to BAT function[94–100]. Hence, it seems plausible to speculate that an appropriate function of the circadian system may be necessary for a correct BAT function, although it remains to be seen.

Furthermore, to understand how different physiological responses oscillate is fundamental to know how circadian clock and disease are related. This will allow us to develop personalised plans, prescribing specific zeitgeber exposures (e.g., timing of light exposure, meals, exercise, or other stimulus such as cold), to maximize the effect of specific interventions on the health outcomes of interest[79]. Interestingly, human studies have shown that heat production (i.e., REE) fluctuates throughout the day (under forced desynchrony protocols)[68,101], together with changes in heat loss, in order to regulate the core body temperature. Similarly, the circadian system plays a dominating role in the morning/evening difference in diet induced thermogenesis (DIT)[102–104], which seems to be independent of the behavioural cycle influence. Nevertheless, it remains unknown whether CIT follows a diurnal/circadian rhythmicity in humans. To know whether CIT varies at different circadian phases (e.g., morning vs. evening), and whether harnessing it at specific time frames could help us to maximize its effects on the daily TEE. The same applies to the metabolic activity of human BAT, whose circadian/diurnal oscillations (if any) also remains unknown.

BEYOND PHARMACOLOGICAL APPROACHES: PHYSICAL ACTIVITY AND EXERCISE AS NEW STRATEGIES TO ACTIVATE BAT

The study of exercise offers enormous potential in the discovery of novel therapies for a wide range of diseases, including cardiometabolic diseases, dyslipidemia, depression, and certain tumours[105]. Exercise leads to a high complex perturbation of homeostasis in a wide number of tissues, largely consequential of the increasing metabolic demands of contracting skeletal muscle. The integrated responses to these demand not only restore homeostasis at short term, but when challenged regularly, produce

adaptations associated with improved health and wellbeing[106]. Indeed, poor exercise capacity (namely cardiorespiratory fitness) is the most powerful predictor of mortality and there is evidence for the prescription of exercise as therapy for many diseases[105,107]. This has been highlighted by previous studies showing that exercise is the real polypill[108], and that it is at least, if not even more effective than some pharmacological treatments to improve certain pathologies[109]. However, the underlying mechanisms by which exercise induces many of its benefits are not completely understood[106].

Exercise has been proposed as a potential stimulus in the recruitment and activation of brown adipose tissue (BAT)[50]. It has been hypothesized that exercise might be able to activate and recruit human BAT through several mechanisms, such as the canonical sympathetic activity increase, and the release of adrenergic independent factors[50,110]. However, experiments are scarce[111–115], and there is not clear evidence yet whether exercise modulates BAT function or not. To date, findings from case control studies[114,116] have shown that trained individuals have lower cold-induced BAT volume and activity than sedentary or untrained individuals, as estimated by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography combined with computed tomography (PET/CT). Similarly, a pilot study[111] found lower BAT activity in trained versus sedentary control participants. Recently, Motiani et al.[117] also showed that 2 weeks of exercise (cycling) decreased insulin-stimulated BAT ^{18}F-FDG uptake in participants with active BAT prior to the intervention. Taken together[111,114,116–119], it seems that exercise is associated with the downregulation of BAT glucose metabolism, although further studies are needed to better understand the exercise-induced adaptations of human BAT.

Importantly, to date, no experiments have examined whether an acute bout of exercise induce changes in the physiological and molecular interface of human BAT. This scientific gap is explained because of the lack of a non-invasive in vivo techniques that allow to monitor BAT activity in real time during exercise, and the complex anatomical characteristics and great plasticity of this tissue[38]. Before trying to translate findings from exercise chronic interventions to humans, it is fundamental to determine if acute exercise can modulate BAT metabolism under well-controlled lab conditions, and if so, through which mechanisms acute exercise is able to modulate it (classical

and alternative mechanisms). A step to initially approach this issue may be performed in rodents, and then translated into humans (whenever possible). Determining which are the underlying mechanisms mediating the effects (if any) of acute exercise on BAT function will help us to further understand the basis of physiological and molecular adaptations to exercise.

Of note, during exercise, the skeletal muscle produces and secretes specific factors, known as myokines, which travel through the blood current to exert its functions in different organs and tissues[120]. It is possible that some of these myokines (e.g., IL-6, meteorin-like, musclin, and irisin, among others) have a potential role modulating BAT activation and recruitment[118,121,122]. Similarly, exercise induces several physiological changes in white adipose tissue, including the release of adipokines (such as leptin or FGF21), that might also modulate BAT activity. In fact, during exercise, the organ cross-talk or communication become even more intense in an attempt to restore energy homeostasis and satisfy all our organisms needs. Therefore, to explore whether these adrenergic independent mechanisms might be modulating BAT activity is crucial. For instance, the release of specific myokines such as IL-6 prompts an increase of the catecholamine secretions, which could provoke changes in BAT activity through the sympathetic-adrenal axis[123], or other vias.

The role of modifiable lifestyle behaviours such as physical activity (PA) on the activation and recruitment of human BAT has been understudied. PA comprises any bodily movement produced by the skeletal muscles which leads to an increase in energy expenditure above rest (i.e., 1.5 metabolic equivalents). Only one study[124] in cancer patients has examined the association between self-reported PA and BAT activity estimated by ^{18}F-FDG-PET/CT, and showed that those participants who reported to be more active had higher levels of BAT activity. Findings of this study are, however, limited due to the fact that it presented several methodological problems that hamper the generalization of its results[125]: firstly, the sample of Dinas et al.[124] study was composed by 40 participants (65% men) with cancer, and it is possible that tumour cells competed with brown and beige adipocytes for ^{18}F-FDG uptake. Secondly, they assessed habitual PA with a questionnaire, which could be biased by social desirability and cognitive challenge. In addition, Dinas et al.[124] performed the ^{18}F-FDG-PET/CT scan in warm conditions, and they quantified BAT using a SUV threshold of 2

(HU, not reported), not following the international recommendations[126]. Therefore, it also remains to be seen whether (objectively measured) physical activity levels under free living conditions could be related to an increased BAT recruitment and activity, determined following the international recommendations.

Similarly, the role of physical activity in the apparition of brown-like adipocytes within WAT (i.e., browning) remains unexplored. Until now, it has been shown that chronic exercise has little or no effect of on the expression of selected browning markers in the abdominal subcutaneous WAT (sWAT)[114,127–129]. However, only a study carried out by the of group Dinas et al.[130,] has examined the role of PA on browning of sWAT. In this study, they showed that healthy adult men who reported moderate intensity PA levels had higher expression of browning markers (PGC-1α, PPARα, or PPARγ) than those who reported low intensity PA levels, in the abdominal sWAT. Nevertheless, this study also presents several limitations. For instance, results cannot be generalized beyond men, they assessed PA with subjective methods, they did not measure specific browning related genes (e.g., TBX1 or TMEM26), etc. Hence, whether objectively measured PA levels are related to the regulation of browning markers in the human sWAT needs to be further addressed.

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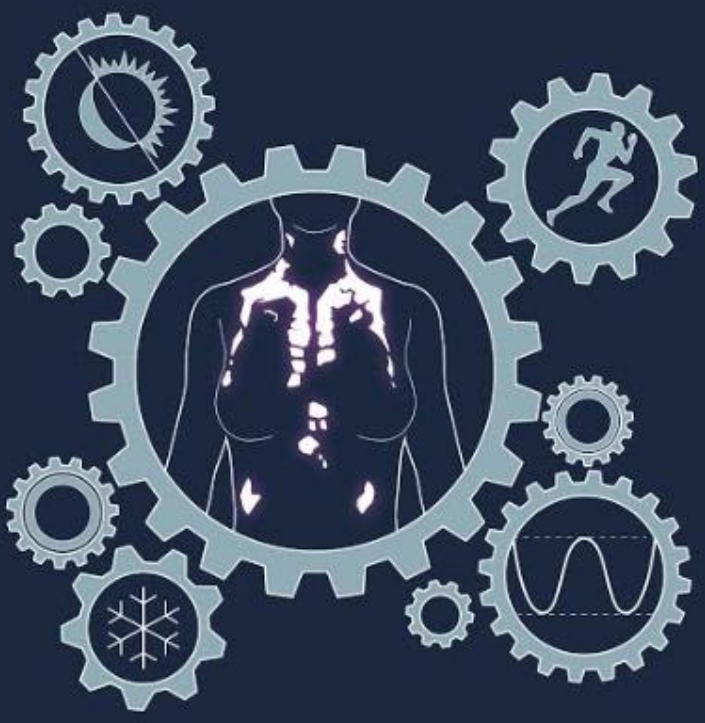
AIMS



AIMS

Overall aim

The overall aim of the present International Doctoral Thesis is to understand the role of exercise, cold, and the biological clock on energy balance in humans as well as on human BAT. This overall aim is addressed in six specific aims which correspond to six different studies:

Part I. Role of cold on energy balance and brown adipose tissue

- **Specific aim I:** to comprehensively describe the physiological responses to an acute bout of mild cold in young lean men - in order to better understand the underlying mechanisms of non-shivering thermogenesis and how it is regulated **(Study I).**

Part II. Role of the biological clock and sleep on energy balance and brown adipose

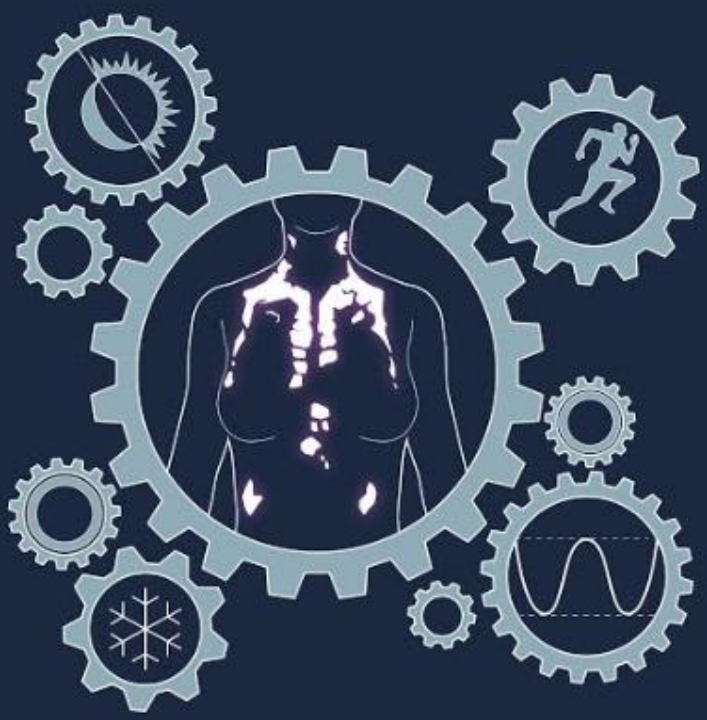
- **Specific aim II:** to examine whether the daily rhythm of distal skin temperature is associated with BAT ^{18}F- F-FDG uptake in young adults; to describe how the personal environmental temperature (during free-living conditions) to which participants are exposed influences BAT volume and activity **(Study II).**
- **Specific aim III:** to examine whether cold-induced thermogenesis and BAT ^{18}F-FDG uptake have diurnal variations **(Study III).**
- **Specific aim IV:** to examine whether sleep duration and quality are related to BAT volume and activity (determined by ^{18}F-FDG uptake) and BAT radiodensity in humans **(Study IV).**

Part III. Exercise and physical activity as new strategies to recruit and activate brown adipose tissue

- **Specific aim V:** to examine whether an acute bout of aerobic exercise affects the protein concentrations of UCP1 and IL-6 in iBAT, as well as to determine whether the IL-6/JAK/STAT3 pathway could be involved in the regulation of UCP1, in wild-type mice **(Study V).**

- **Specific aim VI:** to examine whether the time spent in different physical activity intensities is related to BAT volume and activity (determined by ^{18}F-FDG uptake) as well as to the expression of brown and beige markers in the subcutaneous white adipose tissue, in young adults **(Study VI).**

METHODOLOGICAL OVERVIEW OF THE STUDIES INCLUDED



METHODOLOGICAL OVERVIEW OF THE STUDIES INCLUDED

The present International Doctoral Thesis is composed of a total of six studies. They are classified in three different parts: **Part I** focuses on the effect of mild cold exposure on non-shivering thermogenesis; **Part II** focuses on the relationship of factors related to the biological clock with BAT ^{18}F-FDG uptake and radiodensity; and **Part III** focuses on the search of potential strategies to activate and recruit human BAT and induce browning in the sWAT.

Table 1 shows the methodological overview of all studies included in the present International Doctoral Thesis.

Study	Design; target population	Project	Participants; animals	Predictor/independent variables (instruments)	Dependent variables (instruments)
Study I *Physiological responses to acute cold exposure in young lean men*	Experimental study: lean young men	Reliability study	11 young lean mean age: 23±2 years, BMI: 23.1±1.2 kg/m2, from Granada, Spain	Cold exposure (individualized cooling protocol)	Time to shivering, energy expenditure and nutrient oxidation rates during cold (indirect calorimetry), skin temperature (wireless thermometers), thermal comfort (VAS scales), muscle electrical activity (electromyography), hemodynamics from the forearm and abdominal regions (near infrared spatially resolved spectroscopy), heart rate variability (hear rate monitor)
Study II *Relationship between the daily rhythm of distal skin temperature and brown adipose tissue ^{18}F-FDG uptake in young sedentary adults*	Cross-sectional study; sedentary adults	ACTIBATE	77 young sedentary adults (74% female, age 22±2 years; BMI 25.2±4.8 kg/m^2), from Granada, Spain	Distal skin temperature, and personal environmental temperature (wireless thermometers)	BAT volume and activity (^{18}F-FDG uptake), body composition (DXA), cardiometabolic markers (standard assays), physical fitness (muscular strength and VO_2 max. test), physical activity (accelerometer), chronotype measures (Munich Chronotype Questionnaire)
Study III *Diurnal variations in cold-induced thermogenesis and 18F-FDG uptake in young sedentary adults*	Composed by 2 sub-studies: i) within subject crossover-study; and ii) and	ACTIBATE	17 young healthy adults (7 women, mean age:25±3, BMI: 23.1±2.8 kg/m^2) – within subject-crossover study; 133 young	Cold exposure (personalized cooling protocol) – in the within subject crossover –study;	CIT and CI CHOox and FATox (indirect calorimetry) – in the within subject crossover-study; BAT ^{18}F-FDG uptake and radiodensity (PET/CT scan) - in the cross-sectional study

	cross-sectional study; young adults		sedentary adults (88 women, mean age: 22±2 years, BMI: 24.9±4.8 kg/m^2) – cross-sectional study; Granada, Spain	time of the PET/CT assessment (cross-sectional study)	
Study IV *Sleep duration and quality are not associated with brown adipose tissue volume or activity – as determined by ^{18}F-FDG uptake, in young, sedentary adults*	Cross-sectional study: young sedentary adults	ACTIBATE	118 young healthy adults (69% women, mean age: 21.9±2.2 years, BMI: 24.9±4.7 kg/m^2); Granada, Spain	Parameters related to sleep duration and quality (accelerometry, and PSQI-Pittsburgh Sleep Quality Index quality -PSQI)	BAT ^{18}F-FDG uptake and radiodensity (PET/CT scan), body composition (DXA)
Study V *Effect of an acute bout of aerobic exercise on UCP1 and IL-6 protein concentrations in interscapular brown adipose tissue*	Pilot experimental study; wild-type mice	BATMICE	Non-exercise group (control group, n= 6, weight=24.95g), exercise group (n=7, weight=24.41g)	Non-exercise (control), or exercise (aerobic incremental exercise test in a treadmill)	Protein concentrations of UCP1, PGC1α, p-p38 MAPK, IL-6, IL-6Rα, STAT3, pSTAT3 in the interscapular BAT, and inguinal and gonadal white adipose tissue (only for UCP1 and PGC1α)
Study VI Association of objectively measured physical activity with brown adipose tissue volume and activity in young adults	Cross-sectional study; young sedentary adults	ACTIBATE	130 young sedentary adults (67% women, mean age: 21.9±2.1 years, BMI: 25±4.8 gk/m^2); Granada, Spain	Time spent in different PA intensities and sedentary behaviour (accelerometry)	BAT ^{18}F-FDG uptake (PET/CT scan), expression of browning markers in subcutaneous white adipose tissue (rt-qPCR), and body composition (DXA)

ACTIBATE: activating brown adipose tissue through exercise, BAT: brown adipose tissue, BMI: body mass index, CI: cold induced, CIT: cold induced thermogenesis, CHO_{ox}: carbohydrates oxidation rate, DXA: dual-x energy ray absorptiometry, ^{18}F-FDG : 18F-Fluordeoxyglucose, FAT_{ox}: fat oxidation rate, PET/CT: positron emission tomography combined with computed tomography, PSQI: Pittsburgh sleep quality index, rqPRC: real time quantitative polymerase chain reaction, VO2max: maximum volume of oxygen consumption.

RESULTS AND DISCUSSION

PART I. Role of cold on energy balance and brown adipose tissue

STUDY I

Physiological responses to acute cold exposure in young lean men

ABSTRACT

The aim of this study was to comprehensively describe the physiological responses to an acute bout of mild cold in young lean men (n = 11, age: 23 ± 2 years, body mass index: 23.1 ± 1.2 kg/m^2) to better understand the underlying mechanisms of non-shivering thermogenesis and how it is regulated. Resting energy expenditure, substrate metabolism, skin temperature, thermal comfort perception, superficial muscle activity, hemodynamics of the forearm and abdominal regions, and heart rate variability were measured under warm conditions (22.7 ± 0.2ºC) and during an individualized cooling protocol (air-conditioning and water cooling vest) in a cold room (19.4 ± 0.1ºC). The temperature of the cooling vest started at 16.6ºC and decreased ~ 1.4ºC every 10 minutes until participants shivered (93.5 ± 26.3 min). All measurements were analysed across 4 periods: warm period, at 31% and at 64% of individual´s cold exposure time until shivering occurred, and at the shivering threshold.

Energy expenditure increased from warm period to 31% of cold exposure by 16.7% (P =0.078) and to the shivering threshold by 31.7% (P = 0.023). Fat oxidation increased by 72.6% from warm period to 31% of cold exposure (P = 0.004), whereas no changes occurred in carbohydrates oxidation. As shivering came closer, the skin temperature and thermal comfort perception decreased (all P<0.05), except in the supraclavicular skin temperature, which did not change (P>0.05). Furthermore, the superficial muscle activation increased at the shivering threshold. It is noteworthy that the largest physiological changes occurred during the first 30 minutes of cold exposure, when the participants felt less discomfort.

INTRODUCTION

When humans are exposed to cold, they exhibit mainly two types of physiological responses in order to protect their core temperature. They can i) rely on their body insulative properties by changes in blood perfusion to decrease heat loss; and/or ii) increase their energy expenditure up to 3±5 fold above the resting energy expenditure (REE)[1], in order to counterbalance heat lost (cold induced thermogenesis, CIT)[2].

Human CIT response has been studied broadly using different methods (cold air, water immersions, water perfused garments, ice-blocks, among others), as well as different exposure times[1]. CIT is typically divided into 2 main components: shivering thermogenesis and non-shivering thermogenesis. Shivering thermogenesis increases heat production in response to cold through muscular contractions. It is able to provoke a 5-fold increase in REE in humans, being fundamental to avoid hypothermia and ensure human survival under extreme cold conditions[3–5]. However, shivering thermogenesis is often uncomfortable and fatiguing, and compromises locomotion. On the other hand, non-shivering thermogenesis is triggered mainly by mild cold and can increase REE to an extent of 30%, without inducing a large discomfort[6]. Consequently, it has become an attractive target as a health-promoting stimulus to counteract obesity and related comorbidities[7–10].

Through many decades, researchers have focused mainly on characterizing the metabolic effects of severe cold exposure, especially in shivering thermogenesis [11]. Nevertheless, it remains unclear how non-shivering thermogenesis is regulated and which are the mechanisms involved during mild cold exposure. Important gaps, such as metabolic pathways and fuel selection need to be further examined. Moreover, the relative contribution of different tissues to non-shivering thermogenesis is still to be discerned. Brown adipose tissue (BAT), a thermogenic tissue with the ability to oxidize carbohydrates and lipids and to dissipate energy in the form of heat, seems to play a key role in non-shivering thermogenesis[12–14]. Estimations suggest that cold-activated BAT could account itself for 2.5±5% of the increase in REE[2]. However, there is an important fraction of body heat production that is missing. Recent findings suggest that other tissues such as the skeletal muscle may also play a key role in non-shivering

thermogenesis[7,15], yet more evidence is needed. Moreover, even less is known about the role of subcutaneous white adipose tissue over non-shivering thermogenesis.

For a better understanding of the underlying mechanisms of non-shivering thermogenesis, a comprehensive analysis of the cold induced physiological responses is required. Consequently, in the present study we described the physiological responses to an acute bout of mild cold exposure until shivering occurred in young lean men. Specifically, we analysed the changes in energy expenditure and substrate metabolism, skin temperature and thermal comfort perception, superficial muscle activity, hemodynamics of the forearm and abdominal regions, and heart rate variability. The changes on these variables were analysed at several temperature time points during an individualized cooling protocol, which encompassed the whole spectrum of non-shivering thermogenesis.

MATERIAL AND METHODS

Study participants

A total of eleven Caucasian male adults (age: 23 ± 2 years; body mass index: 23.1 ± 1.2 kg/m^2; lean mass index: 17.1 ± 1.2 kg/m^2; fat mass index: 4.5 ± 0.9 kg/m^2) participated in this experimental trial (ClinicalTrials.gov, ID: NCT02365129). All participants were healthy, non-smokers, and did not take any medication that could have altered their energetic or neuromuscular responses to cold exposure. The study protocol and the informed consent were performed in accordance with the Declaration of Helsinki (revision of 2013). The study was approved by the Human Research Ethics Committee of the University of Granada (nº 924) and of the "Servicio Andaluz de Salud" (Centro de Granada, CEI-Granada). The study was conducted between March and April 2016.

Procedures

The study protocol is shown in **Figure 1.** The participants were advised (i) not to change their sleeping habits, (ii) to refrain from any moderate (within the previous 24 hours) or vigorous physical activity (within the previous 48 hours), and (iii) not to drink alcoholic or stimulant beverages within the previous 6 hours). In addition, they were advised to arrive at the centre in fasting conditions (at least 8 hours). The assessments took place between 8.30h - 16.00h, except in the case of two participants who were assessed between 16.30h - 20.00h. At their arrival, the participants confirmed that

they followed all the pre-study conditions. They voided their bladders and dressed in standardized clothes (sandals, shorts, and T-shirt, clo-value: 0.20). Weight and height were measured (Seca, Hamburg, Deutschland). The participants entered a warm room (22.7 ± 0.2ºC) where they lay on a reclined bed for 40 minutes. They were not allowed to stand up, move or rub their bodies, or to fall asleep. Afterwards, they entered an air-conditioned room (19.4 ± 0.1ºC) and lay on a bed in the same position. Fifteen minutes after entering the cold room, the participants put on a temperature controlled water perfused cooling vest (Polar Products Inc., Ohio, USA), which covered the clavicular region, as well as their chest, abdominals, and back. Water temperature started at 16.6ºC, and decreased ~ 1.4ºC every 10 minutes until shivering occurred. The shivering threshold was determined visually and by asking the participants if they were experiencing shivering. The cold exposure protocol was recorded on video and trained researchers further confirmed shivering onset. The participants lay quietly in the warm room to acclimate to the environmental temperature (22±23ºC) for 20 minutes. Then, from minute 21 to 40, gases exchange parameters, skin temperature, hemodynamics of the forearm and abdominal regions, and heart rate variability were recorded. When the participants were moved into the cold room, the same measurements were made from minute one until shivering occurred. Electromyography was recorded between minutes 31±40 in the warm room, and during the whole cold exposure. We also assessed the thermal comfort perception in both warm and cold conditions.

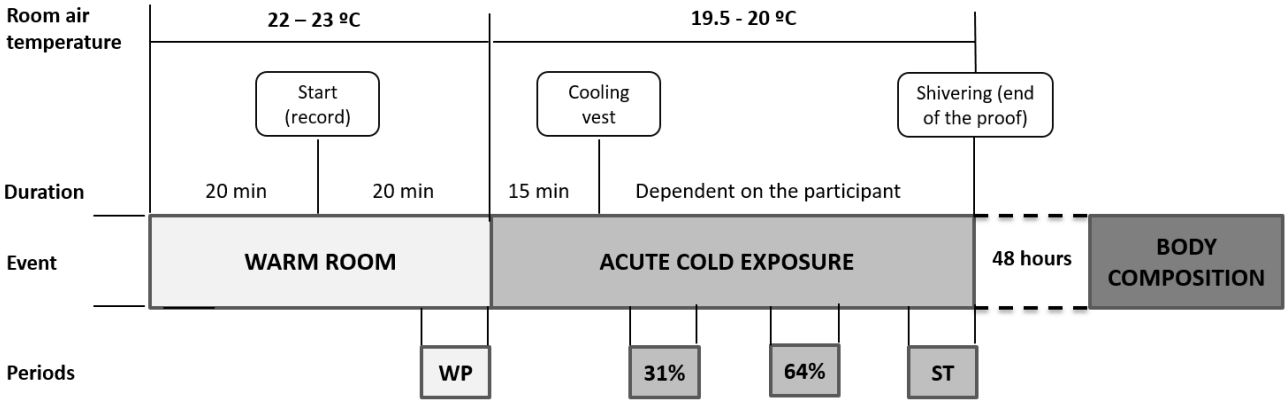


Figure 1. Study protocol

Physiological measurements

Resting energy expenditure and substrate metabolism.

Gases exchange parameters were recorded by indirect calorimetry using a breath-by-breath technique metabolic cart (CCM Express, Medgraphics Corp, St Paul, Minneapolis, USA). A neoprene face-mask equipped with a directconnect™ metabolic low flow sensor (Medgraphics Corp, Minnesota, USA) was used for gases collection. Flow calibration was performed using a 3 L calibration syringe at the beginning of every testing day. Furthermore, the metabolic cart was re-calibrated every 30 minutes using two standard gas concentrations following the manufacturer's instructions.

Raw data were obtained every 10 seconds using the Breeze Suite software (Medgraphics Corp, St Paul, Minneapolis, USA). REE and respiratory quotient were calculated from the readouts of oxygen consumption volume and carbon dioxide production volume. REE was calculated according to the equation proposed by Weir[16], and substrate metabolism was estimated following the equations reported by Frayn[17] and Schadewaldt et al[18].

REE (*kcal=day*) = (1.106 * VCO^2 + 3.941 * VO^2) * 1.44

RQ= VCO^2 /VO^2

Protein Oxidation (PRO Ox) (*g/day*) = (0.15 * *REE)* / 16.74

*Carbohydrate Oxidation (g/day) = [(*4.55 * VCO^2 – 3.21 * VO^2) * 1.44] – 0.459 * PRO Ox

*Fat Oxidation (g/day) = [1.67 * VO^2* -1.67 * VCO^2) * 1.44] – 0.307 * PRO Ox

The changes over cold exposure in REE and substrate metabolism were calculated using the warm period (WP) as baseline.

Skin temperature and thermal comfort perception.

Skin temperature was measured with 26 iButtons (adapted from Schellen et al[19]) (DS-1922 L, Thermochron; resolution: 0.0625ºC; Maxim, Dallas, USA) attached to the skin on different body sites (Supporting information, **Figure S1**). Skin temperature was recorded at 1-minute intervals and the mean[20], proximal[21], and distal skin temperature[22] were calculated. Furthermore, we calculated a peripheral gradient (forearm minus fingertip)[23] for each side of the body as a proxy of peripheral vasoconstriction. We also calculated the difference between the skin temperature of the right side of the chest and the supraclavicular zone as a proxy of BAT activity[24], and the whole-body gradient (distal minus proximal skin temperature)[21]. Equations used to determine the skin temperature parameters are shown in **Table S1**. The analysis of all data recorded by the iButtons and the calculation of equations were carried out with the Temperatus software (http://profith.ugr.es/temperatus). We used visual analogue scales to assess the thermal comfort perception, where 0 mm was “not cold at all” and 100 mm was “maximum tolerable cold” in both warm and cold conditions. The participants reported their thermal comfort perception in the whole body, as well as in their hands, feet, abdomen, and supraclavicular zone.

Superficial muscle activity (Surface electromyography).

Surface electromyography (EMG) wireless electrodes (Trigno Wireless Delsys EMG System, Boston, Massachusetts, USA) were placed on eight muscles (*Vastus Medialis*, *Vastus Lateralis*, *Rectus Femoris*, *Rectus Abdominis*, *Pectoralis Major*, *Deltoid*, *Trapezius*, *and Sternocleidomastoid*) on the right side of the body, following the current recommendations[25]. Raw EMG signals were amplified at a gain of 909 (differential amplifier, 20±450 Hz) and sampled at 2 kHz. A raw EMG data analysis was performed using Matlab (Version R2015a, The Mathworks, Natick, Massachusetts, USA). EMG signals were band-pass-filtered (20±500 Hz, 4th order zero-lag Butterworth filter), and the mean EMG root mean square (RMS) (mV) with a 50-ms moving rectangular window was calculated to determine activation throughout the assessments. The raw EMG RMS (mV) data were used for within-muscle changes in electrical activity comparisons throughout the cold exposure. The number of EMG

activity bursts was also quantified to provide burst shivering rate (BSR) (bursts min-1) as a measure of the possible muscle activation during cold exposure. A shivering burst was defined as an EMG RMS period lasting ≥0.2s at an amplitude greater than the intensity threshold and with a minimum inter-burst duration ≥0.75s[26]. Intensity threshold was calculated by first averaging the EMG RMS activity and then averaging all EMG RMS values remaining above. The periods comprising voluntary movement were excluded from the analysis. The measurements were carried out in 6 out of the 11 participants.

Hemodynamics of the forearm and abdominal regions.

We used a near infrared spatial resolved spectroscopy (NIR_{SRS}) device (Portamon, Artinis Medical System, the Netherlands), a dual wavelength continuous system which simultaneously combines the modified Beer-Lambert and spatial resolved spectroscopy. We attached one device to the left ventral forearm (as representative of muscle tissue), in the medial point between the wrist and elbow joint, and another one to the left side of the abdomen (as representative of subcutaneous white adipose tissue), 2 cm from the umbilicus in the horizontal axis. Portamon provides the absolute value of tissue saturation index expressed as percentages (TSI%), and the relative changes in the concentration of total haemoglobin (ΔtHb), oxy-haemoglobin (ΔO_2Hb), and deoxy-haemoglobin (ΔHHb), expressed in µmol.

Portamon light sources in both positions were situated at 30, 35, and 40 cm from the receptor, which allows a measurement of approximately 4 cm^3 of volume and a penetration depth of approximately 2 cm[27]. Moreover, we assumed constant oxygen independent light losses due to scattering in tissue. A differential pathlength factor of 4 was established for the forearm[28,29] and for the abdomen (arbitrary value). The sample rate was set at 10 Hz and data were analysed with the Oxysof software (Portamon, Artinis Medical System, the Netherlands).

Heart rate variability.

We used a Polar RS800CX (Polar Electro Oy, Kempele, Finland) heart rate monitoring system at 1000 Hz-frequency. The Polar RS800CX wirelessly receives heart rate data from a chest strap worn by participants. The data were analysed with Kubios HRV, version 2.2 software (Kuopio, Finland). We selected a low artefact correction level and applied smooth priors filter method with a lambda = 500 to remove trend

components. Since the RR interval time series is an irregularly sampled series, a cubic spline interpolation rate of 4 Hz was used to convert the RR series into equidistantly sampled. Then, frequency bands were established at 0±0.04 Hz (very low frequency), 0.04±0.15 Hz (low frequency), and 0.15±0.4 (high frequency), and the spectrum estimation was calculated with the Fast Fourier Transformation (window width = 256 s, window overlap = 50%). We deleted the time intervals which did not meet a normal distribution, were unimodal, or presented outliers. The time and frequency domain parameters of heart rate variability were calculated. Concerning time domain parameters, we measured the mean length of all RR intervals, the percentage of consecutive normal RR intervals differing more than 50 ms, the square root of the mean squared sum of the differences of successive NN intervals, and the standard deviation of all RR length intervals. Regarding the frequency domain parameters, we measured the absolute and normalized power of high frequency and low frequency, as well as the low-high frequency ratio. High frequency seems to be an indicator of the parasympathetic nervous system tone, whereas low frequency is thought to be controlled by both sympathetic and parasympathetic systems [30–33]. Low frequency-high frequency ratio has been suggested as a measurement of sympathovagal balance[34], although it remains unclear[35].

Body composition.

Body fat and lean mass were measured by Dual Energy X-ray Absorptiometry (HOLOGIC, QDR 4500 W) 48 hours after the completion of measurements. We also measured adipose tissue thickness at the abdomen and the forearm in triplicate using a skinfold caliper (British Indicators Ltd, UK). The measurements were taken at the same place where the Portamon diodes were placed, since adipose tissue thickness affects in vivo NIRS measurement[36].

Statistical analysis

The data were analysed in 4 time periods (**Figure 1**): (i) WP; (ii) 31% and (iii) 64% of the individual's cold exposure time until shivering occurred; and (iv) shivering threshold (ST). We used several temperature points along the mild cold exposure (31% and 64% of cold exposure and ST) to have a representative period of each third of the individualized cooling protocol. Measurements in each period were an average of 5 minutes. In the WP, we analysed the data of skin temperature, hemodynamics of the

forearm and abdominal regions, and heart rate variability recorded between minutes 36±40. For REE and substrate metabolism analyses, we selected the 5 continuous minutes with the lowest mean coefficient of variance of oxygen consumption and carbon dioxide production volume, respiratory quotient, and minute ventilation between minutes 21±40. Similarly, the 5-minute period selected to analyse EMG was the one with the most stable values between minutes 31±40 during the WP. In the cold period, we analysed the 5 minutes immediately after the 31% or 64% of the individual's time exposed to cold until shivering occurred. Finally, the ST period comprised the previous 5 minutes to shivering onset.

The changes over time in data of normally distributed variables (REE and substrate metabolism, skin temperature, thermal comfort perception, EMG RMS, hemodynamics of the forearm and abdominal regions, and heart rate variability) were analysed with a repeated measures analysis of variance (ANOVA). Pairwise comparisons were performed using the Bonferroni post-hoc tests. The changes over time in EMG BSR were analysed with Friedman Test, since they did not follow a normal distribution. Adjusted significance was chosen for Friedman test. The level of significance was set at $P<0.05$. Statistical Package for the Social Sciences (SPSS, version 22) was used to perform the statistical analysis (IBM, NewYork, USA).

RESULTS

Mean time ± standard deviation at which 31% and 64% of cold exposure and ST started was 28 ± 8 min, 59 ± 17 min, and 88 ± 26 min, respectively, after the beginning of the cold exposure (**Figure 2A**). **Figure 2B** shows the room air and cooling vest temperature across periods.

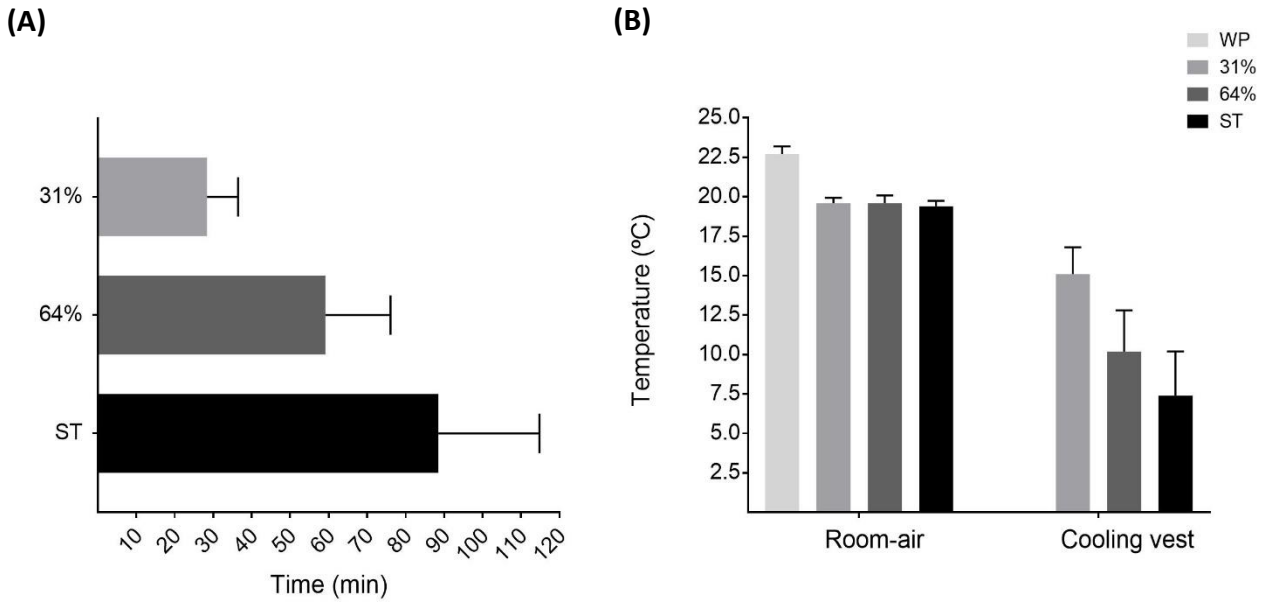


Figure 2. Mean time of the study periods (A), and room-air and cooling vest temperature (B). Values are mean ± standard deviation. ST: shivering threshold, WP: warm period, 31% and 64%: percentage of the individual's time exposed to cold until shivering occurred.

Resting energy expenditure and substrate metabolism

REE increased from WP to 31% [mean difference (95% confidence interval): 263 kcal/day (24,551), P = 0.078] and 64% [235 kcal/day (47, 423), P = 0.014] of cold exposure and ST [500 Kcal/day (64, 936), P = 0.023; respectively] (Figure 3A). However, REE did not show any significant change from 31% and 64% of cold exposure to ST (P>0.05). Fat oxidation increased from WP to 31% and 64 of cold exposure [41 g/day (14, 68), P = 0.004; 40 g/day (13, 67), P = 0.005; respectively], whereas there was no change in carbohydrates oxidation (P>0.05, **Figure 3B** and **3C**).

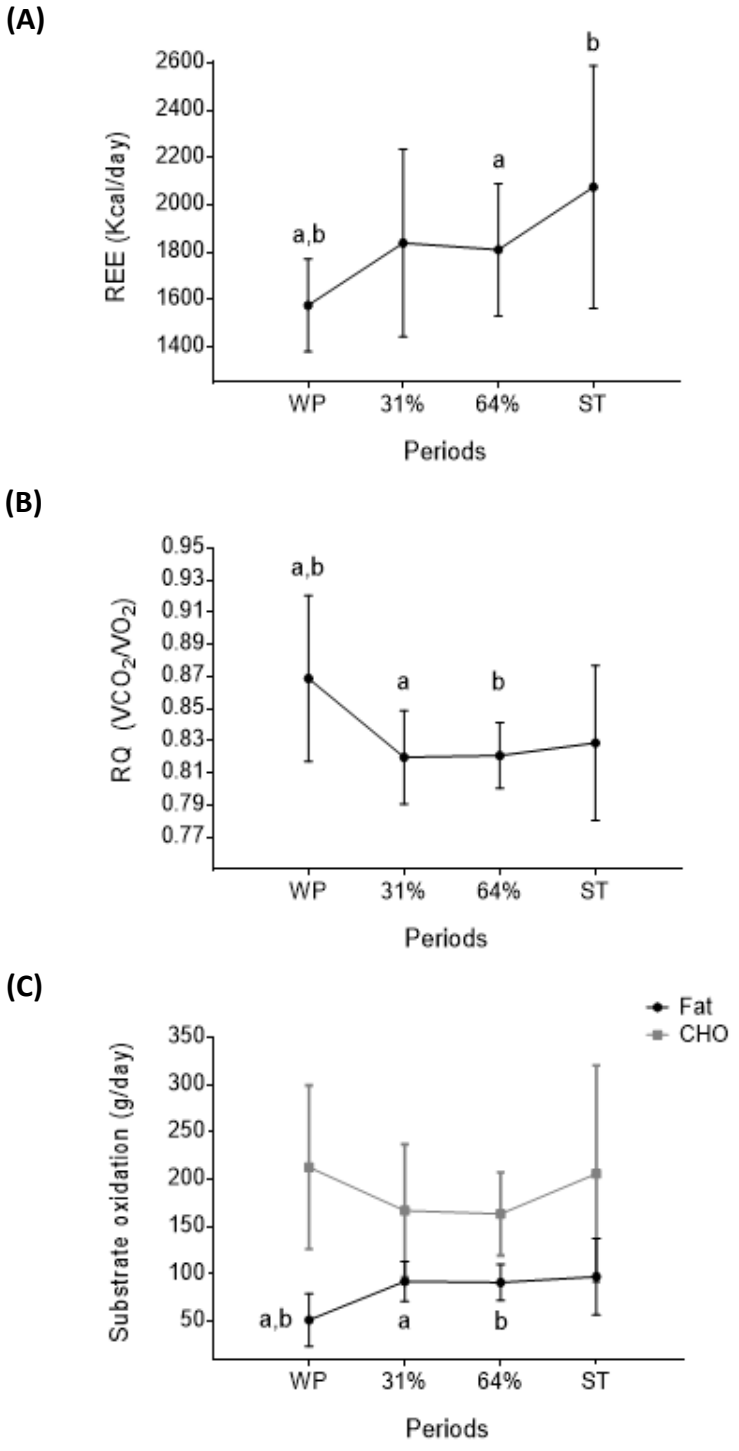


Figure 3. Resting energy expenditure (A), respiratory quotient (B) and substrate metabolism (C) across study periods. Values are mean ± standard deviation (n = 10). Repeated measures analysis of variance was performed, using Bonferroni post-hoc tests for pairwise comparisons. Common letters show significant differences (P_0.05) between two specific periods. CHO: carbohydrates, REE: resting energy expenditure, RQ: respiratory quotient, ST: shivering threshold, WP: warm period, 31% and 64%: percentage of the individual's time exposed to cold until shivering occurred.

Skin temperature

The mean, proximal, and distal skin temperature significantly decreased across all periods (all P≤0.05), whereas the right supraclavicular skin temperature did not show any significant change (P>0.05) (**Figure 4A**). The peripheral gradient (forearm minus fingertip) increased in both arms from WP to 31% and 64% of cold exposure and ST (all P≤0.001) (**Figure 4B**). Moreover, the supraclavicular gradient increased across all periods, whereas the whole-body gradient decreased (all P<0.05, all P≤0.001, respectively; **Figure 4C**).

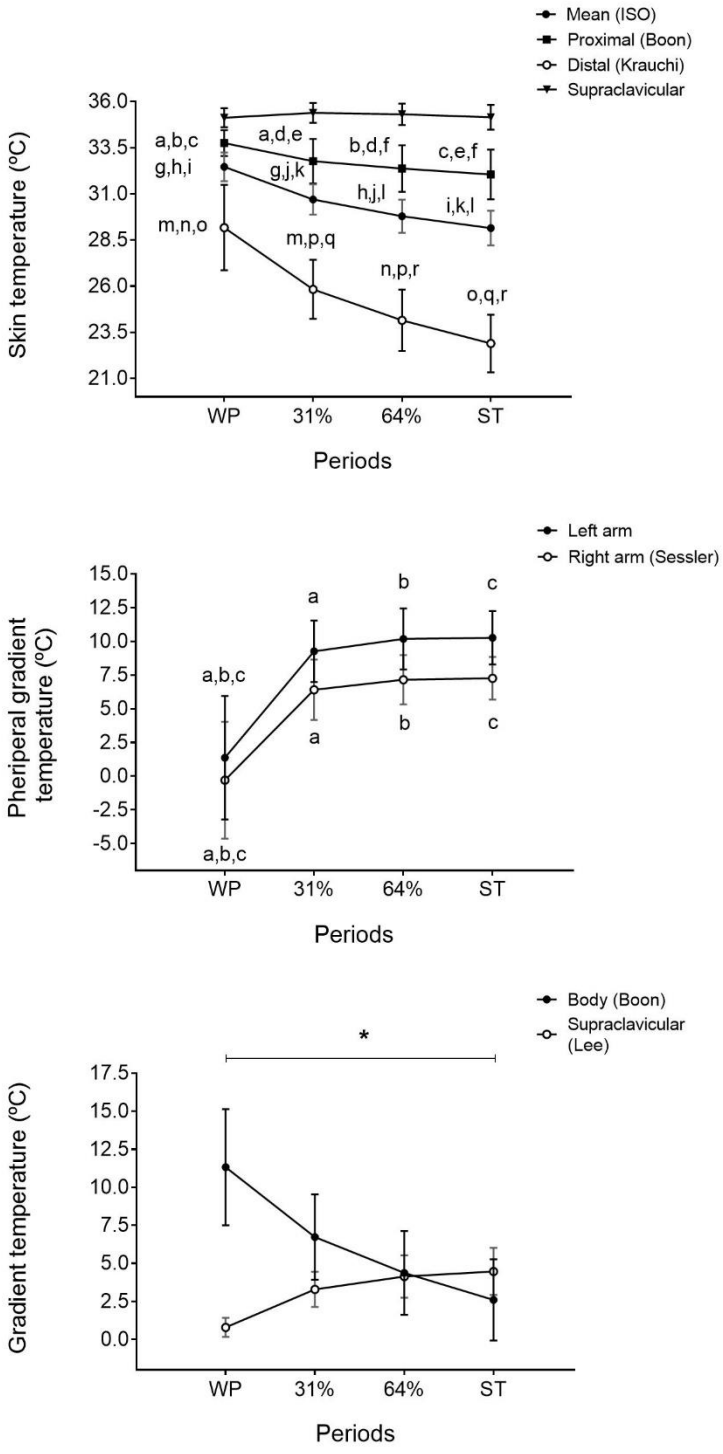


Figure 4. Skin temperature and body gradients across study periods. Panel (**A)**: skin temperature, Panel (**B**): proxies of peripheral vasoconstriction in both arms, Panel (**C**): body and supraclavicular skin temperature gradients. Values are mean ± standard deviation. Repeated measures analysis of variance was performed, using Bonferroni post-hoc tests for pairwise comparisons. Common letters show significant differences ($P<0.05$) between two specific periods. Symbol shows significant differences among all periods ($P<0.05$). ST: shivering threshold, WP: warm period, 31% and 64% percentage of the individual's time exposed to cold until shivering occurred.

Thermal comfort perception

A significant increase in thermal discomfort was observed in the whole body and in each body part reported from WP to 31% and 64% of cold exposure and ST (all P<0.05, Figure 5).

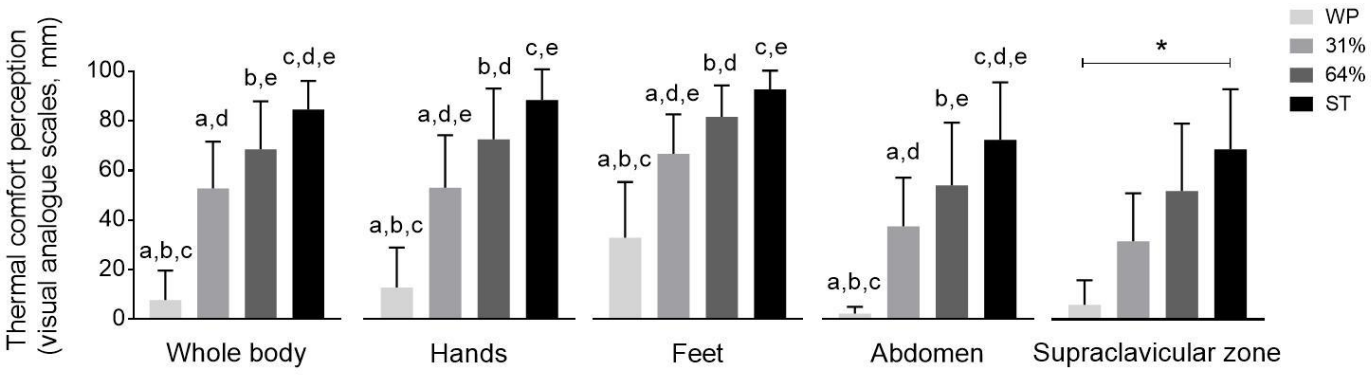


Figure 5. Thermal comfort perception measured by visual analogue scales across study periods. Visual analogue scales measured thermal comfort from "no cold at all" (= 0 mm) to "maximum tolerable cold" (= 100 mm). Values are mean ± standard deviation (n = 11). Repeated measures analysis of variance was performed, using Bonferroni post-hoc tests for pairwise comparisons. Common letters show significant differences between two specific periods (P<0.05). Symbol shows significant differences among all time periods (P<0.05). ST: shivering threshold, WP: warm period, 31% and 64%: percentage of the individual's time exposed to cold until shivering occurred.

Superficial muscle activity (EMG)

RMS did not show significant differences across periods in any muscle (P > 0.05). BSR in *Vastus Lateralis*, *Rectus Femoralis*, *and Pectoralis* showed an increase from WP and 31% of cold exposure to ST (all P<0.05). BSR of *Sternocleidomastoid* increased from WP to ST (P<0.05). The results (n = 6) are shown in **Figure 6.**

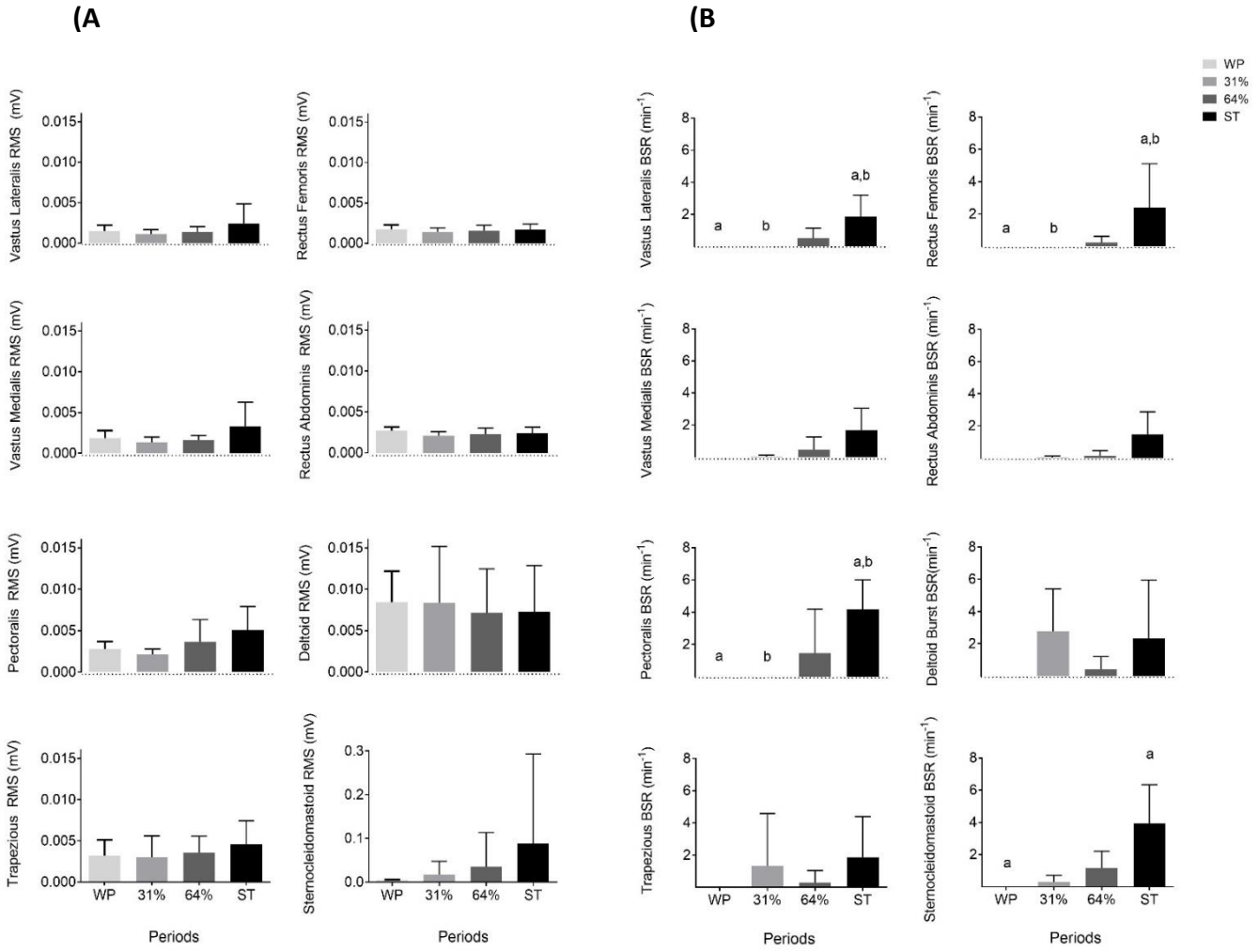


Fig 6. Electrical muscle activity (mV) and burst shivering rate (min-1) of eight different muscles across study periods. Panel (**A**): root mean square, Panel (**B**): burst shivering rate. Values are mean ± standard deviation (n = 6). Repeated measures analysis of variance (Bonferroni post-hoc tests) and Friedman test (adjusted significance) were respectively performed for EMG RMS and EMG BSR. Common letters show significant differences between periods (P < 0.05). BSR: burst shivering rate, RMS: root mean square, ST: shivering threshold, WP: warm period, 31% and 64%: percentage of the individual's time exposed to cold until shivering occurred

Hemodynamics of the forearm and abdominal regions (NIRSRS parameters)

TSI% increased from WP to 31% of cold exposure [2.89% (5.5, 0.3), P = 0.032] in the abdominal region (Figure 7), whereas no differences were found in ΔtHb, ΔO2Hb, and ΔHHb across periods (P>0.05). No changes were observed in TSI%, ΔtHb, ΔO2Hb, and ΔHHb in the forearm region through all periods (P>0.05, **Figure 7).**

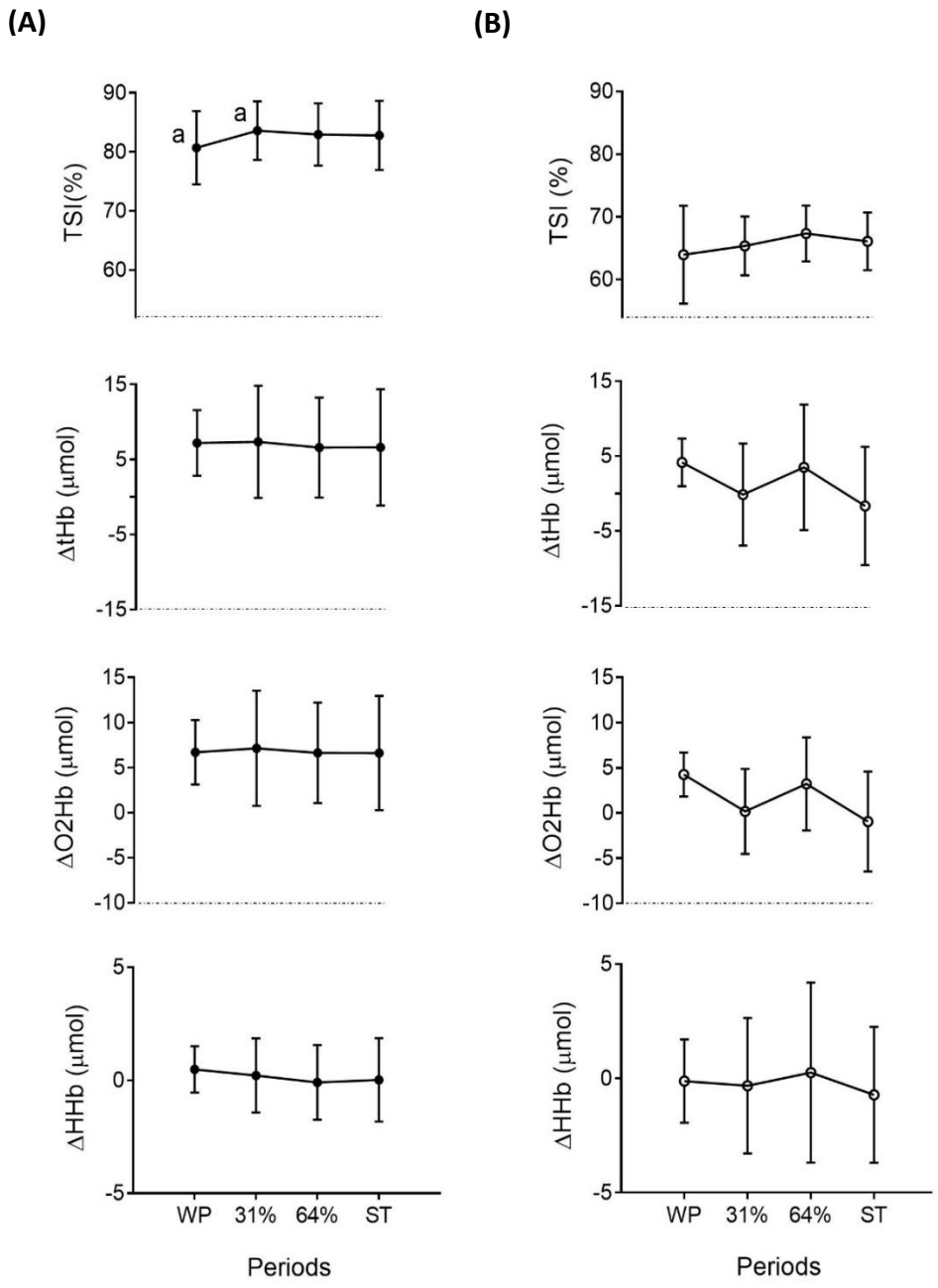


Figure 7. Tissue saturation index (%) and relative changes in the concentration of total haemoglobin (ΔtHb), oxy-haemoglobin (ΔO2Hb), and deoxy-haemoglobin (ΔHHb) in the abdominal and forearm regions across study periods. Panel (**A**): abdominal region, Panel (**B**): forearm region. Values are mean ± standard deviation (n = 9). Repeated measures analysis of variance was performed, using Bonferroni post-hoc tests for pairwise comparisons. Common letters show significant differences between two specific periods (P<0.05). ST: shivering threshold, WP: warm period, 31% and 64%: percentage of the individual's time exposed to cold until shivering occurred.

Heart rate variability

All frequency domain parameters of heart rate variability were similar across periods (P>0.05, **Figure 8).** Furthermore, time domain parameters did not change (P>0.05, **Table S2**).

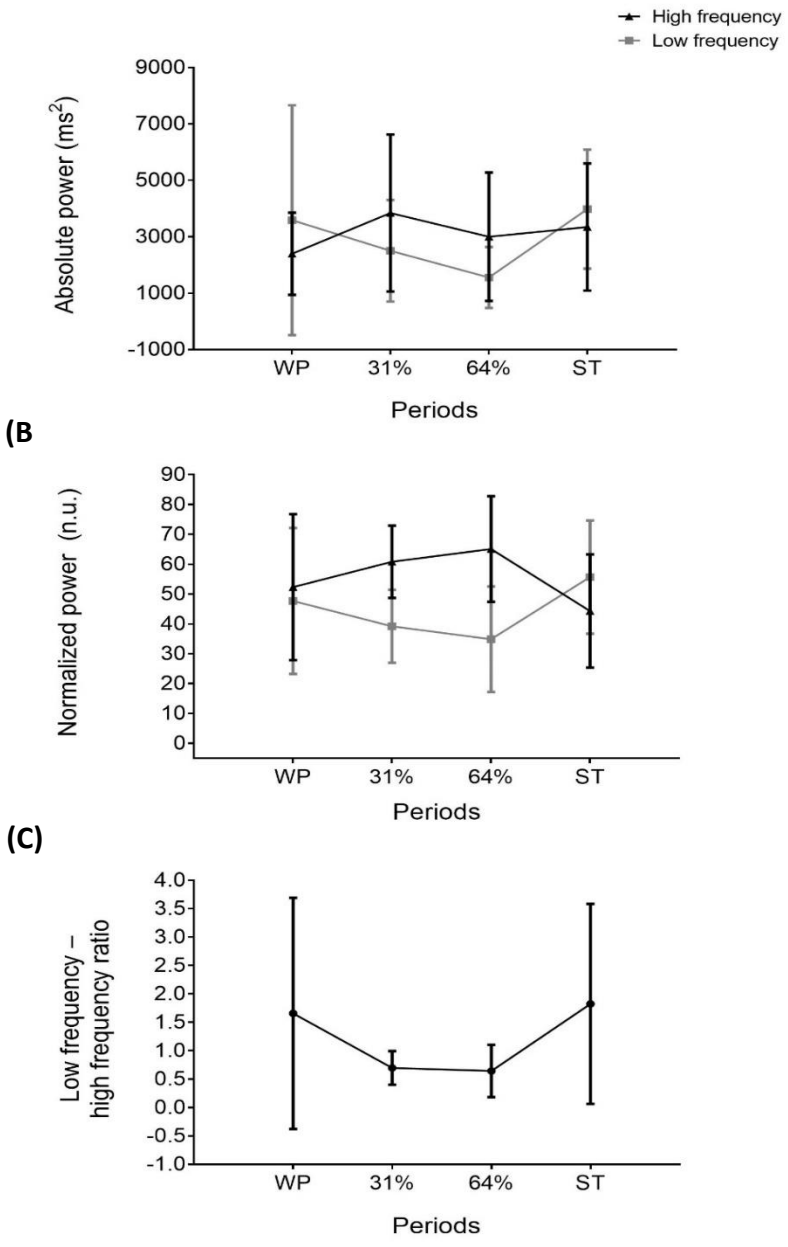


Figure 8. Frequency domain parameters of heart rate variability across study periods. Panel (**A**): absolute power, Panel (**B**): normalized power, Panel (**C**): low frequency-high frequency ratio. Values are mean ± standard deviation (n = 7). Repeated measures analysis of variance was performed, using Bonferroni post-hoc tests for pairwise comparisons. No significant differences were found across study

periods (P>0.05). ST: shivering threshold, WP: warm period, 31% and 64%: percentage of the individual's time exposed to cold until shivering occurred.

DISCUSSION

In the present study, we extend previous findings on the potential of mild cold exposure to increase REE and to prompt fat-oxidative metabolism in young lean men. As shivering came closer, skin temperature (mean, proximal and distal) and thermal comfort perception decreased, except the supraclavicular skin temperature, which did not change. Furthermore, superficial muscle activation increased in the ST, when shivering was reported by the participants and visually determined by the researchers. It is noteworthy that the largest physiological changes occurred between WP and 31% (≈30 minutes) of the individual's cold exposure time, when the participants reported less discomfort.

Shivering threshold

Most studies examining the effect of acute cold exposure on human metabolism have used a standardized and non-individualized cooling protocol (e.g. similar duration, intensity, and type of cold stimulus for every participant), in order to make experiments replicable. However, when non-individualized cooling protocols are applied, inter-individual differences are not considered, which is likely to affect the cold induced physiological responses. Furthermore, given the fact that non-shivering thermogenesis shows a high inter-individual variability[1], to individualize the cooling protocol to every participant becomes relevant. The complex nature of shivering makes it difficult to determine its onset and regulation in humans[37]. In this experiment, “shivering threshold” was used as the end-point of the cooling protocol, in which shivering was self-reported by the participants and visually determined by the researchers. The use of a self-reported shivering threshold might not be considered as a valid method to establish shivering onset, since conscious thermal perception and localization are regulated by the thalamus and cerebral cortex, whereas shivering is controlled by the preoptic area of the hypothalamus[5,37]. In addition, the visual determination of shivering threshold by researchers is not an objective measurement. Hence, the shivering threshold was only considered an indicator of shivering onset that allowed us to individualize the cooling protocol for every participant. Nevertheless, to the best of our knowledge, no studies have compared yet whether the use of a

subjectively determined shivering threshold differs from the onset of shivering determined by EMG. To note also is that shivering might be a combination of both voluntary and involuntary muscular contractions, and consequently EMG could also present inaccuracies in determining the onset of shivering. Furthermore, although other methods such as temperature clamping have been proposed[38], the use of shivering threshold has been extensively used and accepted as a valid method to maximize non-shivering thermogenesis and activate BAT[21,39–43]. Most important, the participants from this study underwent the same cooling protocol 48 hours after, replicating all conditions, and the time at which shivering threshold took place did not change (unpublished observations). Consequently, despite its limitations, shivering threshold may a potential and reliable method to determine shivering onset.

Resting energy expenditure and substrate metabolism

We observed a mean total increase in REE of 31.7% since the participants were exposed to mild cold until they shivered, which concurs with previous literature[1,2]**.** The increase in REE from WP to 31% of cold exposure accounted for a large part of total REE increase (16.7%). Regarding substrate metabolism, previous studies[44,45] have shown that mild cold exposure is likely to induce an increase in fat oxidation, whereas carbohydrate oxidation decreases. In line with these reports, we observed a large increase in fat oxidation (72.6%) from WP to 31% of cold exposure, whereas CHO remained unchanged along the cold exposure. To note is that a plateau on REE and substrate metabolism was observed from 31% to 64% of cold exposure, which could indicate an initial adaptation to cold or a shift in the metabolic pathway. Taking all findings together, it seems that non-shivering thermogenesis accounted for a large part of CIT, especially during the initial moments of cold exposure, that is around the first 30 minutes in our cooling protocol. Furthermore, superficial muscle increased its shivering activity during the last minutes of cooling, clearly contributing to the increase of REE. Although shivering thermogenesis has been reported to increase REE up to 5 times[3,4], we observed a lower increase in REE since our cooling protocol was designed to finish at the onset of shivering.

Skin temperature and thermal comfort perception

Skin temperature decreased in most of the measured body anatomical points along the cold exposure, but the skin temperature of the supraclavicular zone did not. The

skin temperature at the supraclavicular zone is an indirect marker of BAT activity or volume during cold exposure [21,46,47]. Therefore, it is plausible that BAT accounted for a part of non-shivering thermogenesis.

This assumption is supported by the increase of the supraclavicular gradient, which is an indirect marker of the heat loss capacity of the supraclavicular zone[24]. In addition, an increase in the gradient of the right arm used as a proxy of peripheral vasoconstriction[23] above 4ºC indicated that a cold-induced peripheral vasoconstriction took place from WP to 31% of cold exposure [48,49]. The largest skin temperature changes were observed from WP to 31% of cold exposure, the period of cold exposure in which the participants felt less discomfort either in the whole body or in each body part.

Superficial muscle activity

As expected, a general increase in superficial muscle activation was observed as shivering came closer, noticing an increase in the BSR from the WP and 31% of cold exposure to ST. Although changes were not statistically significant ($P>0.05$), most muscles had a minimum or noticeable burst shivering rate during 64% of cold exposure when shivering was not reported by participants or visually detected by researchers. This finding suggests that tests designed to analyse the non-shivering thermogenesis and that are not controlled by electromyography are partially influenced by superficial muscle activity. The increase of CIT across cold exposure may also be explained by the shivering activity of deeper central-located muscles, which was not determined in this study[15]. Further studies should quantify both superficial and deep muscle activity by electromyography during cold exposure to understand better the actual contribution of muscle to CIT. Of note is also that deltoid showed a different trend in shivering to the rest of muscles, showing a high muscle BSR during the 31% of cold exposure. This trend seemed to be cause of voluntary movement by the participants rather than the thermal strain caused by the cooling protocol.

Hemodynamics of forearm and abdominal regions (NIR_{SRS} parameters)

The use of NIRS to study changes in oxidative metabolism during cold exposure is relatively recent[50,51]. Several authors have used NIRS parameters such as regional blood oxygen saturation as a proxy of tissue oxidative metabolism[50], and total haemoglobin as an index of blood volume or tissue vasculature[51]. However, the use of

different types of devices and the lack of quantification hampers inter-studies comparisons. In the present study, TSI% (an indicator of the oxygen saturation of regional vasculature) increased from WP to 31% of cold exposure in the abdominal region (used as a proxy of subcutaneous white adipose tissue oxygenation), whereas ΔtHb (an index of blood volume) did not change. Consequently, since blood volume was constant and oxygen saturation of regional vasculature increased, abdominal subcutaneous white adipose tissue oxidative metabolism decreased from WP to 31% of cold exposure. This finding suggests that subcutaneous white adipose tissue is not involved in the observed increase of REE during cold exposure.

Heart rate variability

Cold exposure increases sympathetic nervous system activity, inducing norepinephrine release and prompting a range of physiological responses as well as brown adipose tissue activation[52–54]. Consequently, it was of interest to determine whether heart rate variability parameters will change in response to mild cold, and more specifically, whether low frequency-high frequency ratio would increase since it has been proposed as an indirect marker of sympathovagal balance[34]. Studies focusing on the effect of acute cold exposure over heart rate variability are scarce. Several experiments have suggested that cold exposure is related to changes in the autonomic sympathetic response[55] or to a higher sympathetic nervous system predominance over parasympathetic system in humans[56]. Nevertheless, in the present study we did not find significant changes in any heart rate variability parameter, probably due to the high inter-individual variability of these parameters. Furthermore, there is still controversy regarding whether the low frequency-high frequency ratio actually reflects sympathovagal balance[35], which could explain why no changes in this parameter were either observed.

Comprehensive insight

There is controversy regarding the underlying mechanisms of CIT during mild cold exposure. Whether shivering or non-shivering thermogenesis act together or independently, and to what extent each component contributes to CIT still remains unclear[1,2]. Several authors have proposed BAT as one of the main mediators of non-shivering thermogenesis[13,14], whereas others suggest that muscle is more predominant[7,15]. It has also been postulated that BAT and muscle contribute

synergistically to non-shivering thermogenesis[57]. Less is known, however, about the role of subcutaneous white adipose tissue over non-shivering thermogenesis. Despite the need of more evidence, we observed that the largest increase of non-shivering thermogenesis in humans would normally happen during initial moments of cold exposure, as we observed in the 31% of cold exposure. Since BAT is mainly fuelled by triglycerides obtained by BAT intracellular lipolysis and plasma non esterified fatty acids[12,13,15,58], the large increase in fat oxidation (72.6%) from WP to 31% of cold exposure suggests that BAT is active.

The decrease of skin temperature in most measured anatomical points, but not in the supraclavicular zone, supports this assumption. These findings add further evidence indicating that BAT acts as a non-shivering thermogenesis effector during mild cold exposure[13]. However, as previously mentioned, the skeletal muscle has also been postulated as a possible contributor to non-shivering thermogenesis and might account for a part of non-esterified fatty acids clearance, especially in proximal deep muscles such as "Longus colli"[15]. Skeletal muscle seems to mainly contribute to glucose turnover, even when shivering is minimized[15]. Nevertheless, the increase in fat oxidation is such that it is not plausible to think that BAT itself could account for all of it. This leads to the idea that muscle increases its fat oxidative metabolism via mitochondrial uncoupling[7] or by low intensity shivering[59]. Hence, BAT and muscle might contribute to non-shivering thermogenesis synergistically, especially at the beginning of mild cold exposure. In addition, the plateau observed in REE and substrate metabolism from 31% to 64% of cold exposure, followed by changes in the trends of substrate metabolism (despite not being statistically significant) might indicate a shift in the metabolic pathway. This metabolic shift could reflect changes in the relative contribution of BAT and skeletal muscle to non-shivering thermogenesis[5], being BAT contribution higher at the beginning when thermal stress was lower, and increasing muscle contribution (and consequently CHO oxidation) as shivering came closer. UCP1 and other brown fat cell genes, characteristic of beige adipocytes, have shown to be prominent in subcutaneous white adipose tissue depots of rodents[60]. Thus, we hypothesized that subcutaneous white adipose tissue would increase its oxidative metabolism during cold exposure as shown by Muzik et al.[50]. However, the oxidative metabolism of the abdominal region seemed to decrease. This might be

explained by the fact that abdominal subcutaneous white adipose tissue depots seem to present a higher resistance to browning[60–62] or that they mostly represent depots of pure white adipocytes. In any case, caution must be paid since NIRS measurement is only a proxy of oxidative metabolism and has not been validated yet in subcutaneous white adipose tissue. Despite the fact that mild cold exposure has been suggested to play a central role counteracting obesity at long term[2,11,63], we only observed a small increase in CIT (~20.83 kcal/h), and consequently its contribution to cause a negative energy balance may be negligible. Nevertheless, mild cold exposure seems to have an important role in counteracting body fat accumulation and in obesity related comorbidities[7–10]. Finally, it is noteworthy that the largest physiological changes in metabolism and thermoregulation occurred during the initial moments of the cold exposure. This occurred specifically from WP to 31% (first≈30 minutes) of the cold exposure, when the participants showed the lowest discomfort perception across cold exposure. This finding provides practical guidelines for future uses of mild cold as a health promoter stimulus, so that the effect of cold in a short time can be maximized while increasing adherence.

A limitation to consider in the present study is that despite the use of the supraclavicular skin temperature as a surrogate marker of BAT activity or volume[21,46,47], we cannot exclude that it is in fact registering the temperature of large blood vessels (i.e. aorta) close to the skin in this area. In addition, we did not measure core temperature, which might have provided useful information to determine whether mild cold exposure actually elicited any thermal benefit. It is also noticeable that the use of superficial EMG only allowed us to study the contribution of superficial muscles to CIT. Regarding NIR_{SRS} parameters, the in vivo scattering properties of the biological tissues and the unknown contribution of myoglobin to the NIRS signal were inherent limitations. On the other hand, we provide a comprehensive insight of the physiological changes that occur during an acute bout of mild cold exposure, using an individualized cooling protocol designed to determine the shivering threshold. We used several temperature points along the cold exposure (31%, 64% of cold exposure and ST) to better analyse the physiological changes through the whole spectrum of non-shivering thermogenesis. This fact is noteworthy, since experiments normally consider only 2 different conditions (warm and cold), lacking important information[1].

In conclusion, non-shivering thermogenesis seems to develop an important role increasing CIT during mild cold exposure, being accompanied by a higher fat oxidative metabolism. Both skeletal muscle and BAT might contribute synergistically to NST increase, whereas the subcutaneous white adipose tissue does not seem to be a key player. Furthermore, we observed that the largest physiological changes occurred during the first 30 minutes of cold exposure, when the participants felt less discomfort during the cold exposure. However, more evidence is needed to understand the underlying mechanisms of non-shivering thermogenesis during mild cold exposure.

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SUPPLEMENTARY MATERIAL

Outcome	Reference	iButtons (n)	Anatomical positions. Fig. S1	Equations
Mean skin temperature	14 ISO 9886-2004 [1] *(14-ISO)*	14	From 1 to 14	(Forehead*0.07)+(Neck*0.07)+(Right Scapula*0.07)+(Left Chest*0.07)+(Right Deltoides*0.07)+(Left Elbow*0.07)+(Right Abdomen*0.07)+(Left Hand*0.07)+(Left Lumbar *0.07)+(Right Thigh*0.07)+(Left Harmstring*0.07)+(Right Shin bone*0.07)+(Left Gastrocnemius*0.07)+(Right Instep*0.07)
Proximal skin temperature	Boon et al. [2]*(Boon)*	3	10,16,8	(Right Thigh*0.383)+(Right Clavicular*0.293)+(Right Abdomen*0.324)
Distal skin temperature	Kräuchi et al. [3] *(Krauchi)*	2	9, 14	(Left Hand+Right Instep)/2
Body temperature gradient	Boon et al. [2]*(Boon)*	5	9,14,10,16,8	[(Left Hand+Right Instep)/2]- [(Right Thigh*0.383)+(Right Clavicular*0.293)+(Right Abdomen*0.324)]
Supraclavicular temperature gradient	Lee et al. [4]*(Lee S-RC)*	2	15, 25	(Right Supraclavicular(S)- Right Chest (RC))

Peripheral temperature Gradient	Sessler et al. [5] Right arm	2	20, 21	(Right Forearm-Right Top of forefinger)

Table S1. Equations used to measure skin temperature. Table adapted from Martinez-Tellez et al.[6]

	WP			31%			64%			ST		
Mean RR (ms)	1064.9	±	81.8	1086.0	±	105.2	1069.9	±	112.1	1025.4	±	121.6
SDNN	70.1	±	26.2	76.4	±	22.5	70.6	±	20.9	81.6	±	21.3
RMSSD (ms)	78.0	±	21.2	91.9	±	31.1	88.6	±	34.3	88.1	±	37.2
pNN50 (%)	50.6	±	88.6	57.1	±	5.8	54.6	±	15.2	46.6	±	17.5

Table S2. Time domain parameters of heart rate variability rate across study periods.
Values are mean ± standard deviation (n = 7). Repetead measures analysis of variance was performed, using Bonferroni correction for pairwise comparisons. No significant differences were observed across periods (P>0.05). Mean RR: mean length of all RR intervals, pNN50: percentage of consecutive normal RR intervals differing more than 50 ms, RMSSD: square root of the mean squared sum of the differences of successive NN intervals, SDNN: standard deviation of all RR legnth intervals, ST: shivering thereshold period, WP: warm period, 31% and 64%: percentage of the individual's time exposed to cold until shivering occurred.

Figure S1

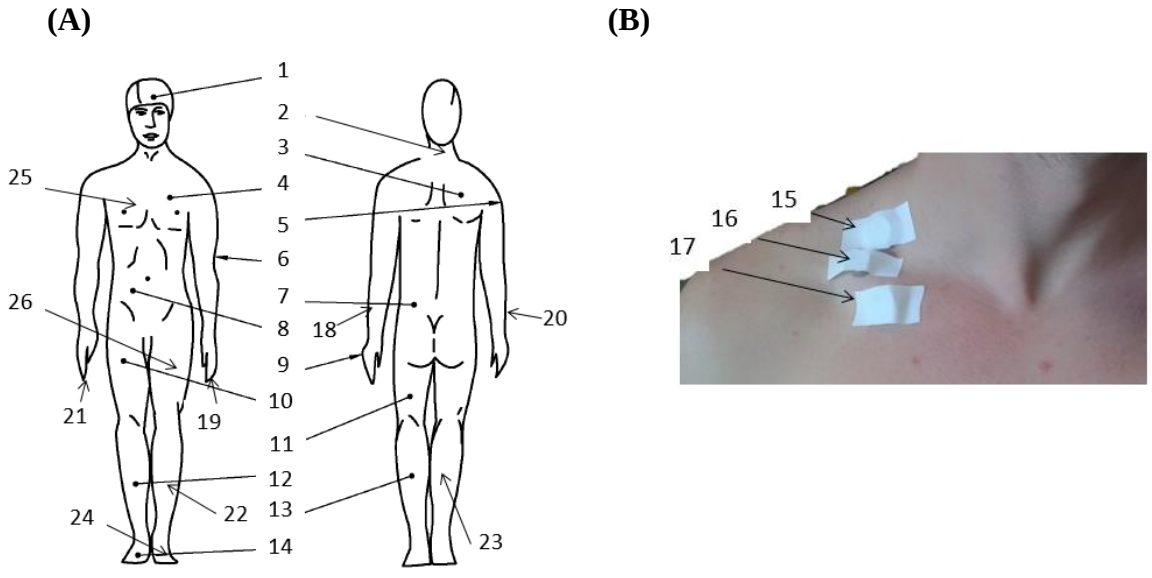

Figure S1. Body anatomical points where iButtons attached to skin. 26 different positions can be distinguished. Panel (**A**): distribution of the iButtons over whole body, Panel (**B**): distribution of the iButtons on the right clavicular sites.

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PART II. Role of the biological clock and sleep on energy balance and brown adipose tissue

STUDY II

Relationship between the daily rhythm of distal skin temperature and brown adipose tissue ^{18}F-FDG uptake in young sedentary adults

ABSTRACT

The present study examines whether the daily rhythm of distal skin temperature (DST) is associated with BAT metabolism as determined by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake in young adults. Using a wireless thermometer (iButton) worn on the non-dominant wrist, DST was measured in 77 subjects (26% male; age 22±2 years; BMI 25.2±4.8 kg/m^2) for seven consecutive days. The temperatures to which they were habitually exposed over the day were also recorded. The interday stability of DST was calculated from the collected data, along with the intraday variability and relative amplitude, the mean temperature of the 5 and 10 consecutive hours with the maximum and minimum DST values respectively, and when these hours occurred. Following exposure to cold, BAT volume and mean and peak standardized ^{18}F-FDG uptake (SUV_{mean} and SUV_{peak}) were determined for each subject via static ^{18}F-FDG positron emission/computed tomography scanning. Relative amplitude, and the time at which the 10 consecutive hours of minimum DST values occurred, were positively associated with BAT volume, SUV_{mean} and SUV_{peak} ($P \leq 0.02$), whereas the mean DST of that period was inversely associated with the latter BAT variables ($P \leq 0.01$). The interday stability and intraday variability of the DST were also associated (directly and inversely, respectively) with BAT SUV_{peak} ($P \leq 0.02$ for both). All these associations disappeared, however, when the analyses were adjusted for the ambient temperature to which the subjects were habitually exposed. Thus, the relationship between the daily rhythm of DST and BAT activity estimated by ^{18}F-FDG uptake, is masked by environmental and likely behavioural factors. Of note is that those participants exposed to the lowest ambient temperature showed 3-5 times more BAT volume and activity compared to subjects who were exposed to a warmer ambient temperature.

INTRODUCTION

Almost all living things have a circadian time-keeping system[1]. The circadian system consists of a network of hierarchically organized structures that regulate the body's temporal organization in relation to its[2–5]. From an evolutionary perspective, this biological clock has allowed organisms to adapt their metabolic processes and their behaviour to cyclic environmental change[4,6,7]. In humans, current social habits commonly disrupt this system (chronodisruption), leading to metabolic problems such as obesity, dyslipidemia or impaired glucose tolerance, perhaps even shortening our life span[2–5]. Certainly, the correct functioning of the circadian system seems necessary for properly and temporally adjusted behavioural and physiological functions, and *vice versa*.

Brown adipose tissue (BAT) is a specialized thermogenic organ, mainly activated during exposure to cold. It produces heat via the action of uncoupling protein 1 (UCP1)[8–10]. Given its 'energy burning' capacity, its endocrine function[11], and its contribution to metabolic homeostasis through the uptake of energy substrates, the BAT is seen as a potential therapeutic target in the fight against obesity and diabetes[10,12–15]. Consequently, much effort is being invested in understanding how human BAT function is physiologically regulated, and how to safely exploit its functions.

Interestingly, in mice it has been shown that the formation and metabolic function of BAT are under circadian clock regulation[6,16,17]. The BAT transcriptome is robustly rhythmic[18,19], with nearly 8% of the tissue's expressed genes showing circadian rhythmicity. Accordingly, it has been shown that a number of key nuclear receptors involved in circadian rhythmicity, such as Rev-erb alpha and PER2, play essential roles in the modulation of murine UCP1 expression and BAT thermogenesis[16,20]. In addition, experiments in rodents have shown that BAT receives inputs from different hypothalamic nuclei, shaping the rhythms of systemic glucose and lipids as well as those of body temperature and energy expenditure[6,21–24]. Although there is growing evidence that the circadian rhythms of body temperature and metabolism are intimately related to a proper BAT function in rodents[25], evidence in humans is scarce. Lee et al. [26] recently suggested that glucose utilization by human BAT is coupled to heat production in a circadian manner. Whether BAT function is

associated with the appropriate functioning of the circadian system, however, remains to be seen.

Several "marker rhythms" have been proposed as a means of assessing circadian system functioning. These marker rhythms - driven by the suprachiasmatic nucleus (SCN, the major pacemaker) - allow the timing of internal biological processes to be followed. Marker rhythms need to be easy to measure in a non-invasive manner, be reliable, have a large amplitude, and have a specific phase relationship with the SCN[7]. Salivary melatonin or cortisol, the rest-activity pattern and core temperature are among the most commonly used[7]. Distal skin temperature (DST) also provides a marker rhythm. This is supported by the fact (among others) that an increase in DST coincides with the onset of the release of dim light melatonin (DLMO)[27], and that it may lead to vasodilation (inducing heat loss) and an eventual fall in core body temperature that helps initiate sleep[28]. In addition, the DST can be easily and comfortably recorded for long periods, is driven by the circadian clock via the autonomous nervous system, and its rhythm persists under demasking analytical conditions[29].

The aim of the present work was to examine whether the daily rhythm of the DST, used as an indicator of the appropriate functioning of the circadian system, is associated with BAT volume and activity as determined by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake in young, healthy adults. The relationship between the DST rhythm and skeletal muscle ^{18}F-FDG uptake was also examined, as was that between disruptions of the DST daily rhythm (more flattened, fragmented, less stable) and obesity and cardiometabolic risk.

MATERIALS & METHODS

The present cross-sectional study was performed within the framework of the ACTIBATE study [30] (in ClinicalTrials.gov, ID: NCT02365129). The study subjects were 82 young healthy (27% male) adults (see enrollment flow chart in Figure S1). All were recruited via advertisements in electronic media and via leaflets. The inclusion criteria required subjects to be 18-25 years old, to be sedentary (self-reported <20 min of moderate-vigorous physical activity on <3 days/week), not to smoke or take any medication that might impact their cardiovascular or thermoregulatory responses to

cold exposure, to have had a stable body weight over the last 3 months (changes <3 kg), to have no cardiometabolic disease (e.g., hypertension, diabetes, etc.), and to have no history of cancer among first-degree relatives.

Assessments were performed at our installations in Granada (southern Spain) between October and November 2016, in four waves involving approximately 20 subjects per wave (Table S1). The study was approved by the Human Research Ethics Committee of the University of Granada (nº 924) and the *Servicio Andaluz de Salud,* and performed in accordance with the Declaration of Helsinki (revision of 2013). All subjects provided written, informed consent to be included.

Distal skin temperature

DST was measured using an iButton DS-1922 L Thermochron wireless temperature sensor (resolution 0.0625°C) (Maxim, Dallas, TX, USA); subjects wore the sensor continuously for 7 consecutive days[31]. The sampling frequency was set at 10 min. The sensor was placed on the ventral area of the non-dominant wrist (over the radial artery), secured with a wristband. Subjects were instructed to remove it only when bathing. Non-wear time periods for each day were recorded in a dairy. Recordings were programmed to begin at 6:00 a.m. and to finish 7 days later at 12:00 a.m (the morning when the ^{18}F-FDG-PET/CT scan was performed; see below).

Distal skin temperature processing

All the non-wear time periods were excluded from analysis. Atypical data were also eliminated by suppressing the time points for which the rate of change with respect to the previous value was higher than the interquartile distance between quartiles 1 and 3 (percentiles 25 and 75, respectively)[32]. Those subjects with under 4 valid days (<75% of the data registered in 24 hours) of data were excluded from analyses. All sensors were programmed, and their data analyzed, using Temperatus® software (http://profith.ugr.es/temperatus?lang=en).

The interday stability (IS) of the DST readings, i.e., the constancy of the 24 h rhythmic pattern over the 7 days of data collection, and the intraday variability (IV), i.e., the fragmentation of the rhythm, and the relative amplitude (RA), were then determined, as described elsewhere[33,34]. RA was determined as the difference between the mean DST for the 5 consecutive hours with the maximum DST values (M5) and the mean DST for the 10 consecutive hours with the minimum values (L10),

divided by their sum[35]. Finally, the times at which L10 and M5 occurred (TL10 and TM5, respectively) were calculated as previously described[35] **(Figure 1A).** The mean daily pattern for DST was calculated per individual, and then the mean for each group calculated. **Figure 1A** shows a representative example of the daily pattern of the DST. The midsleep timepoint, chronotype and social jet lag of the participants were also recorded (see Supplementary Material for more information).

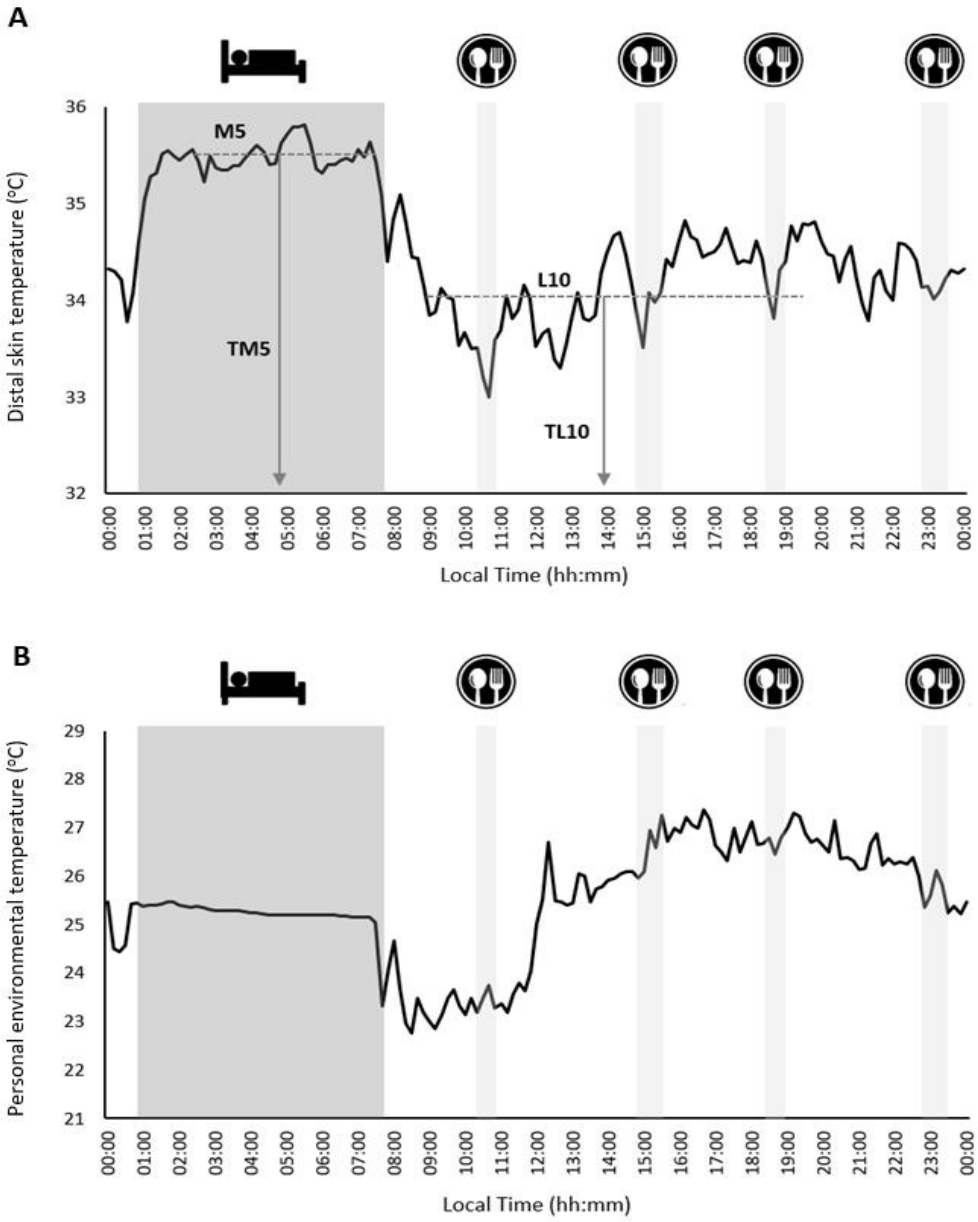

Figure 1. Representative example of the daily pattern of distal skin temperature **(Panel A)** and personal environmental temperature **(Panel B)**. The darkest filled area represents the sleep timetable, and the clearest filled areas represent the times of breakfast, lunch, a snack and dinner. Panel A summarizes the analysis of the distal skin temperature, showing the M5 (average for the 5 consecutive hours with the maximum values), L10 (average for the 10 consecutive hours with the minimum values), and the times at which these occurred (TM5 and TL10, respectively).

Personal environmental temperature

Relationships have been reported to exist between the environmental temperature to which people are exposed and the presence, volume and activity of BAT as estimated by ^{18}F-FDG-PET/CT[36,37]. Since the study period was October to November 2016, the possible confounding effect of environmental temperature on the association between the DST and BAT ^{18}F-FDG uptake was examined[31]. People normally spend around 90% of their time indoors, especially in colder regions/seasons[38], rendering outdoor ambient temperature an inaccurate measure of true exposure. Thus, at the same time the DST assessments were made, the personal environmental temperature (personal-ET) to which participants were exposed was objectively measured using a hip-worn iButton (not covered by clothing). This sensor was never in direct contact with the body[39]. During periods of sleep, the sensor was placed on the bedside table. For a more extended explanation see[31]. The processing, cleaning and analysis of the personal-ET data was performed in the same way as for the DST variables (see above). The daily patterns for the personal-ET were then characterized as for DST. Figure 1B shows a sample personal-ET daily pattern. Finally, based on theoretical concerns and preliminary statistical analyses (data not shown), the mean personal-ET over the L10 period (personal-ET_{L10}) was deemed to be a potential confounder of the examined relationships.

Personalized cold exposure and ^{18}F-FDG-PET/CT acquisition

Full details of how the subjects were exposed to 'personalized cold' and how the ^{18}F-FDG-PET/CT data were acquired can be found elsewhere[40,41]. Briefly, subjects came to the lab and sat in a cool room (19.5-20ºC) wearing a water-perfused cooling vest (Polar Products Inc., Stow, OH, USA). The water temperature was reduced from 16.6ºC by ~1.4ºC every 10 min until they began to shiver (visually detected or self-reported); this value was recorded as the shivering threshold temperature. Some 48-72 h later, the subjects went to the Hospital Virgen de las Nieves, where they were again placed in a cool room (19.5-20ºC) and wore the same cooling vest but with the water temperature set ~4ºC above their shivering threshold temperature for 2 h. After the first hour of cold exposure, the subjects received an injection of ^{18}F-FDG (180.6 ± 5.8 MBq, ≈2.9 MBq/kg) and the water temperature was increased by 1ºC to avoid shivering. One hour later the PET/CT scan was performed using a Siemens Biograph 16 PET/CT

scanner (Siemens, Erlangen, Germany). A low dose CT scan (120 kV) was first performed for attenuation correction and anatomic localization. Immediately thereafter, one static acquisition of 2 PET bed positions (6 min each) was performed from the atlas vertebra to the mid chest region[40]. All personalized cold exposure treatments and ^{18}F-FDG-PET/CT data acquisitions were performed according to current methodological recommendations[42].

^{18}F-FDG-PET/CT analysis

The BAT volume and metabolic activity, estimated via the 18F-FDG uptake, were then determined using the Beth Israel plug-in for the FIJI program43. This required: 1) outlining regions of interest (ROIs) in the supraclavicular, laterocervical, paravertebral and mediastinal regions from the atlas vertebra to the 4th thoracic vertebra, using a 3D-axial technique; 2) the determination of the number of pixels in the above ROIs with a radiodensity range of -190 to -10 Hounsfield Units; and 3) the calculation of individualized, standardized threshold ^{18}F-FDG uptake values (SUV) [1.2/(lean body mass/body mass)][42]. BAT volume was determined as the number of pixels in the above range with an SUV value above the SUV threshold. BAT activity was represented as the mean SUV (SUV_{mean}: the mean quantity of ^{18}FDG in the above same pixels) and peak SUV (SUV_{peak}: the mean of the three highest ^{18}F-FDG contents in three pixels within a volume of <1 cm^3). BAT metabolic activity was calculated as BAT volume x SUV_{mean}. The mean BAT radiodensity was calculated as the mean HU value for the above mentioned ROIs.

In addition, a single slice-ROI was used to determine the ^{18}F-FDG-uptake in several skeletal muscles (*cervical, scalene, longus colli, paravertebral, subscapular, sternocleidomastoid, supraspinous, trapezius, deltoid, pectoralis major, and triceps braquii*), on both the right and left sides of the body. The mean SUV_{peak} for both sides of the body for the deep (*cervical, scalene, longus colli, paravertebral, subscapular*) superficial (*sternocleidomastoid, trapezius, deltoid, pectoralis major, triceps braquii*), and all skeletal muscles or areas, was then determined. Skeletal muscle was distinguished from adipose tissue since skeletal muscle has a radiodensity range of between 10-100 HU. The SUV_{peak} for the descending aorta (reference tissue) at the height of the 4th thoracic vertebra was also determined, using a single ROI from one slice. Finally, for confirmatory analyses, the BAT SUV_{mean} and SUV_{peak}, and the all-

muscles and descending aorta SUV_{peak} with respect to lean body mass (SUV_{LBM})[44] were calculated.

Anthropometry and body composition

Subjects' weight and height (without shoes and wearing the standard clothes) were determined using a SECA model 799 electronic column and scale (SECA, Hamburg, Germany). Waist circumference was measured twice (and the mean determined) at the minimum perimeter; when subjects had abdominal obesity, measures were taken just above the umbilicus, in the horizontal plane. Body composition was measured by dual X-ray absorptiometry using a Discovery Wi apparatus (HOLOGIC, Bedford, MA, USA). Subject body mass indices (BMI) were calculated as *weight (kg) divided by height squared (m^2)*, fat mass index (FMI) as *weight (kg) of body fat / height squared (m^2)*, and lean mass index (LMI) as *lean body mass (kg) / height squared (m^2)*.

Cardiometabolic profile

To assess the cardiometabolic profile of the subjects, and to examine how it related to their DST variables, a set of glycemic and lipid markers were measured (see Supplementary Material) along with insulin, C-reactive protein, the systolic and diastolic blood pressure at rest, and physical fitness (muscular strength and cardiorespiratory fitness). The value for the homeostatic model assessment (HOMA) of insulin resistance was also determined, and the prevalence of metabolic syndrome calculated. More information on how these variables were measured and calculated is provided in the Supplementary Material.

Statistical analysis

Descriptive statistics for continuous and categorical variables were used to show the sociodemographic and clinical characteristics of the study participants. Since the interaction sex x DST variables had no significant influence on the BAT ^{18}F-FDG uptake variables ($P>0.05$), all the analyses were conducted for men and women together. Simple linear regressions were first performed to examine the association between the DST variables and BAT volume, SUV_{mean}, and SUV_{peak}. Multiple linear regression analyses were then performed to examine this relationship after adjusting for sex, and for sex plus personal-and SUV_{peak} were then calculated and the Mann-Whitney test (exact significance) with Bonferroni correction, used to examine differences across BAT tertiles. Linear regression was also used to examine the association between DST

variables and skeletal muscle and descending aorta ^{18}F-FDG uptakes, before and after adjusting for sex and personal-ET_{L10}. Linear regression analysis was further used to determine whether the DST variables

were associated with body composition and subject cardiometabolic profile, before and after adjusting for sex and personal-ET_{L10}. Statistical significance was set at $P \leq 0.05$. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS v.24, Inc. Chicago, IL, USA).

RESULTS

From among the 82 subjects with complete DST and ^{18}F-FDG assessments, three subjects were excluded due to problems with their BAT analyses. Two more were excluded because they had under 4 valid days of sensor measurements **(Figure S1).** The final study sample was therefore made up of 77 subjects (26% male). **Table 1** shows the descriptive characteristics of these subjects by sex. Subjects wore the wrist sensor for 6.1±0.5 days at 21±0.2 h/day.

	All (n=77)*	Men (n=20)	Women (n=57)
Age (years)	23 (2)	22 (2)	22 (2)
Professional status, n (%)			
Student	37 (49)	10 (50)	27 (49)
Unemployed	27 (36)	6 (30)	21 (38)
Other professional activities	11 (15)	4 (20)	7 (13)
Anthropometry and body composition			
Waist circumference (cm)	81.3 (14.4)	94.6 (15.7)	76.7 (10.6)
BMI (kg/m^2)	25.2 (4.8)	28.4 (5.6)	24.1 (3.9)
LMI (kg/m^2)	14.4 (2.3)	17.2 (2.2)	13.4 (1.4)
FMI (kg/m^2)	9.4 (3)	9.6 (3.8)	9.3 (2.8)
Body fat (%)	37.5 (7)	33.6 (8.2)	38.9 (6)
VAT mass (g)	340.3 (191.3)	488.7 (205)	288.2 (157.5)
Cardiometabolic profile			
Glucose (mg/dL)	88 (7)	91 (10)	87 (6)
Insulin (µIU/mL)	8.78 (6.60)	11.72 (10.80)	7.73 (3.85)
Total cholesterol (mg/dL)	167 (36)	161 (44)	169 (33)
High density lipoprotein cholesterol (mg/dL)	53 (12)	44 (7)	56 (11)
Low density lipoprotein cholesterol (mg/dL)	97 (29)	96 (33)	98 (27)
Triglycerides (mg/dL)	89 (62)	110 (79)	81 (53)
HOMA index	1.99 (1.79)	2.81 (3.03)	1.69 (0.92)
C-reactive protein (mg/L)	2.7 (3.6)	2.7 (2.7)	2.7 (4)
Systolic pressure (mmHg)	115.3 (12.4)	126.3 (12.3)	111.6 (10.1)
Diastolic pressure (mmHg)	70.2 (8.3)	71.8 (11.3)	69.6 (7.1)
Muscular strength (kg)	31.1 (7.8)	41.1 (7.3)	27.5 (3.9)
Cardiorespiratory fitness (mL/kg/min)	40.7 (7.6)	42.4 (9.8)	40.1 (6.7)
Metabolic syndrome prevalence (%, ATP III and IDF)	3 (4)	3 (16)	0 (0)
Overall physical activity (mG/5 s)	32.22 (8.98)	29.88 (8.99)	33.07 (8.90)
Chronotype measures			
Midsleep timepoint (hh:mm)	04:56 (00:58)	05:09 (01:02)	04:51 (00:56)
Chronotype (hh:mm)	05:18 (01:24)	05:22 (01:12)	05:17 (01:29)
Social jet lag (hh:mm)	01:21 (00:52)	01:13 (01:04)	01:24 (00:47)
Distal skin temperature variables			
Valid days (days)	6.1 (0.5)	6.1 (0.6)	6.1 (0.5)
Wear time (hours/day)	21 (1.8)	21 (2.1)	21 (1.7)
IS	0.45 (0.13)	0.40 (0.15)	0.47 (0.12)
IV	0.18 (0.10)	0.25 (0.13)	0.16 (0.08)
RA	0.03 (0.02)	0.03 (0.02)	0.04 (0.02)
TL10 (hh:mm)	16:24 (02:54)	15:51 (03:50)	16:36 (02:31)
TM5 (hh:mm)	04:27 (01:57)	03:51 (02:43)	04:39 (01:35)
L10 (°C)	33 (1.1)	32.7 (1.4)	33.1 (1)
M5 (°C)	35.3 (0.7)	34.7 (0.5)	35.5 (0.6)
^{18}F-FDG PET/CTvariables			
BAT SUV threshold	2.10 (0.23)	1.97 (0.22)	2.14 (0.21)
BAT radiodensity (HU)	-58.56 (11.81)	-54.98 (11.37)	-59.78 (11.80)
BAT volume (mL)	69.52 (61.47)	87.76 (78.3)	63.12 (53.73)
BAT SUV_{mean}	3.71 (1.90)	3.50 (1.42)	3.79 (2.04)
BAT SUV_{peak}	11.05 (8.46)	10.53 (8.07)	11.24 (8.66)
Superficial muscles SUV_{peak}	0.61 (0.15)	0.62 (0.11)	0.60 (0.17)
Deep muscles SUV_{peak}	1.12 (0.34)	1.14 (0.36)	1.11 (0.33)
All muscles SUV_{peak}	0.85 (0.22)	0.86 (0.20)	0.85 (0.22)
Descending aorta SUV_{peak}	1.60 (0.35)	1.76 (0.37)	1.55 (0.33)

Table 1. Characteristics of the study participants. Continuous variables are presented as means (standard deviation), and categorical variables as numbers (percentages). *Data were missing for: professional status (remaining cases, n=75), glycemic and lipid markers, HOMA index and C-reactive protein (n=76), systolic and diastolic blood pressure (n=75), cardiorespiratory fitness (n=76), metabolic syndrome prevalence (n=74), resting metabolic rate (n=72), overall PA (n=75), chronotype measures (n=73), BAT radiodensity (n=75). ATP III: National Cholesterol Education Program Adult Treatment Panel III, BAT brown adipose tissue, BMI body mass index, FMI fat mass index, HOMA homeostatic model assessment for insulin resistance, HU Hounsfield units, IDF International Diabetes Federation, IS interday stability, IV intraday variability, LMI lean mass index, L10 mean of the 10 consecutive hours with the lowest values and when they occurred (TL10), M5 mean of the five consecutive hours with the highest values and when they occurred (TM5), PA physical activity, RA relative amplitude, SUV standardized uptake value, VAT visceral adipose tissue.

Association between DST variables and BAT ^{18}F-FDG uptake

Simple linear regression showed RA and TL10 to be directly associated with BAT, SUV_{mean}, and SUV_{peak} (all P≤0.02, see **Figure 2C and D**), whereas L10 was inversely associated with them (all P≤0.01, see **Figure 2F**). Furthermore, IS was directly, and IV inversely, associated with BAT SUV_{peak} (all P≤0.04**, Figure 2A and B**). RA, TL10 and L10 explained the greatest amount of variance in the BAT volume (~8-12%), SUV_{mean} (~5-10%) and SUV_{peak} (~8-14%) values. Neither the midsleep timepoint, chronotype nor social jet lag (see Supplementary Material) were associated with the BAT volume, SUV_{mean}, or SUV_{peak} (all P>0.05, data not shown).

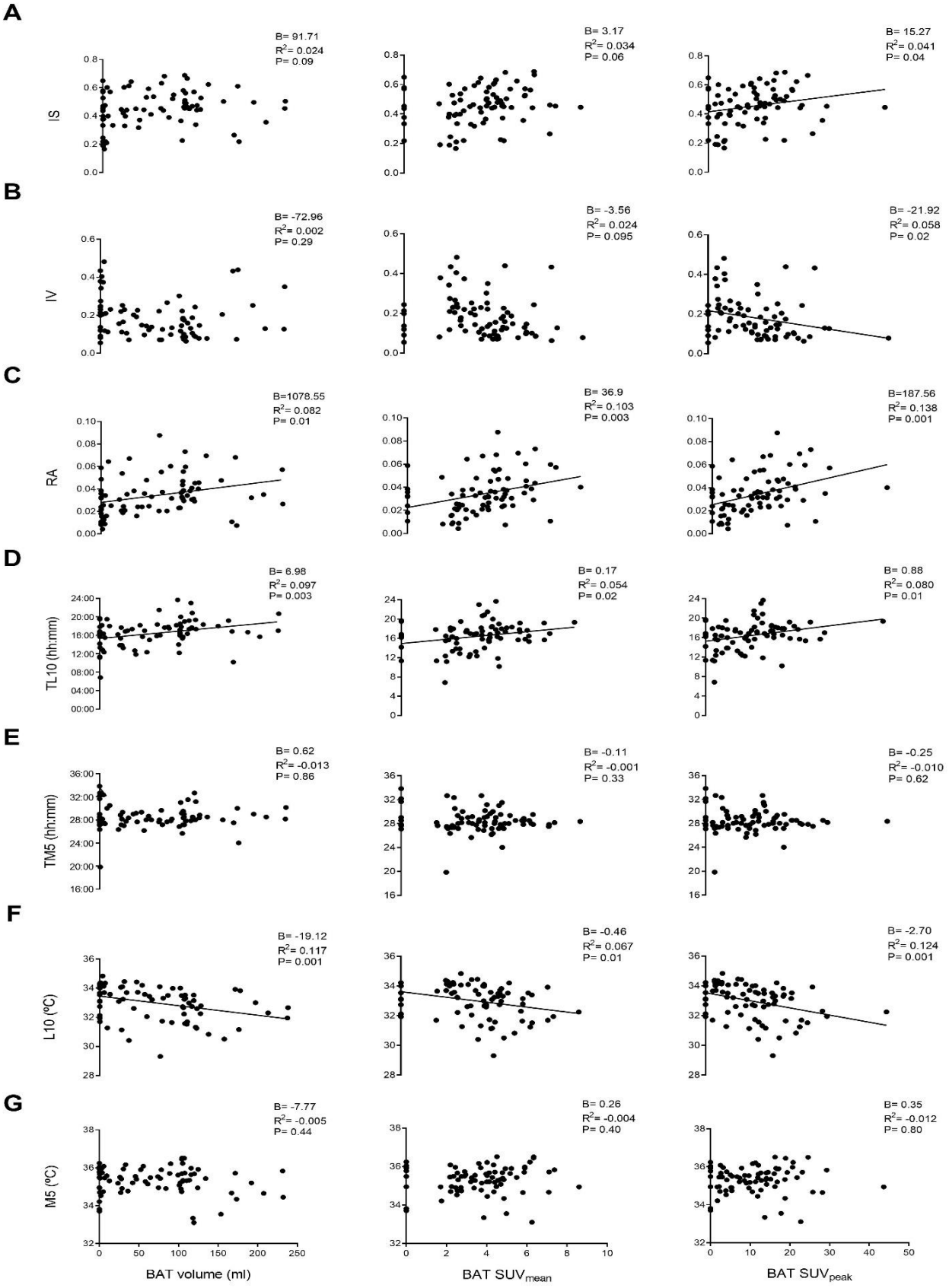

Figure 2. Association of distal skin temperature (DST) variables with brown adipose tissue (BAT) volume and standardized uptake values (SUV) (mean and peak) (n=77) *as determined by simple linear regression). The non-standardized B coefficient, adjusted R^2 and P values are provided. IS interday stability, IV intraday variability, L10 mean of the 10 consecutive hours with the lowest values and when

they occurred (TL10), M5 mean of the five consecutive hours with the highest values and when they occurred (TM5), RA relative amplitude.

These results persisted when the analyses were adjusted for sex, except for the relationship between IS and BAT volume, which became significant (P=0.03; see **Table 2,** Model 1). When these analyses were adjusted for sex plus personal-ET_{L10}, all these associations disappeared (all P>0.05, see **Table 2,** Model 2), but an inverse relationship between TM5 and BAT SUV_{mean} (P=0.04) appeared. Overall, these results remained similar when the Model 2 analysis was additionally adjusted for the time that the participants wore the sensor (data not shown), for BMI, LMI, FMI or percentage fat (see Table S2), and when the evaluation wave (natural day of the year in which the PET/CT scan was performed) was taken into account instead of the personal-ET_{L10} (data not shown). The daily PA level had no influence on the latter relationship (data not shown; for further information, see Supplementary Material).

Table 2. Association between distal skin temperature (DST) variables and brown adipose tissue (BAT) volume/standardized uptake value (SUV; mean and peak) after adjusting for potential confounders.

	BAT volume (mL)			**BAT SUV_{mean}**			**BAT SUV_{peak}**		
	B	R^2	P	B	R^2	P	B	R^2	P
Model 1 (adjusted for sex)									
IS	123.12	0.068	**0.03**	3.13	0.021	0.07	15.75	0.029	**0.04**
IV	-134.37	0.049	0.07	-3.63	0.011	0.12	-24.40	0.05	**0.02**
RA	1185.52	0.119	**0.03**	36.59	0.092	**0.003**	188.62	0.127	**0.001**
TL10 (hh:mm)	7.50	0.132	**0.002**	0.166	0.043	**0.03**	0.884	0.068	**0.01**
TM5 (hh:mm)	1.68	0.008	0.65	-0.124	-0.006	0.28	-0.29	-0.021	0.57
L10 (ºC)	-18.11	0.121	**0.003**	-0.49	0.067	**0.01**	-2.81	0.121	**0.001**
M5 (ºC)	0.129	0.005	0.99	0.23	-0.017	0.53	0.169	-0.025	0.92
Model 2 (adjusted for sex and personal-ET_{L10})									
IS	295.79	0.281	0.34	0.75	0.236	0.65	4.56	0.270	0.52
IV	-16.33	0.272	0.81	-0.09	0.234	0.96	-8.38	0.273	0.37
RA	323.14	0.278	0.42	10.76	0.241	0.40	70.46	0.282	0.20
TL10 (hh:mm)	4.26	0.306	0.06	0.07	0.244	0.32	0.43	0.284	0.18
TM5 (hh:mm)	-1.64	0.274	0.65	-0.23	0.277	**0.04**	-0.77	0.290	0.12
L10 (ºC)	-5.94	0.282	0.32	-0.12	0.238	0.52	-1.16	0.285	0.16
M5 (ºC)	-2.92	0.273	0.77	0.10	0.235	0.75	-0.40	0.266	0.77

Linear regression analyses were performed to examine the association between DST variables and BAT ^{18}F-FDG uptake. Model 1 was adjusted for sex (n=77), and Model 2 for sex and the mean personal environmental temperature over the L10 period (personal-ET_{L10}, n=76). The non-standardized B coefficient, adjusted R^2 and P-value are provided. Significant values are shown in bold (P≤0.05). IS: interday stability, IV: intraday variability, L10: mean of the 10 consecutive hours with the lowest values and when they occurred (TL10), M5: mean of the five consecutive hours with the highest values and when they occurred (TM5), RA: relative amplitude.

DST variables and daily rhythms across BAT volume and activity tertiles

Figure 3A and B show the values for the DST variables and daily patterns with respect to BAT volume tertiles. Compared to the low BAT volume tertile members, subjects in the high BAT volume tertile showed greater IS and RA in their DST patterns, a lower L10, and a phase delay in TL10 (all P≤0.011). In addition, the DST pattern was less fragmented (IV) (P=0.017). No significant differences were seen across tertiles with respect to M5 or TM5 (P>0.017). Similar results were recorded for the DST variables and mean daily patterns with respect to BAT metabolic activity (*BAT volume x* $SUV_{mean,}$ **Figure 3D and E**) and SUV_{peak} tertiles (**Figure 3G and H**). However, RA was also different between the low and medium tertiles (P<0.017), and no significant differences in TL10 were seen across the BAT SUV_{peak} tertiles (P>0.017). It is noteworthy that the subjects exposed to the lowest personal-ET throughout the day and night (high BAT tertile) had approximately 3-5 times greater BAT volume, metabolic activity, and SUVpeak values than those exposed to the highest personal-ET (low BAT tertile) (BAT volume >104.07 vs. 0-27.06 mL; BAT metabolic activity >474.36 vs. 0-90. 63; BAT SUV_{peak} >14.1 vs. 0-5.38; see **Figure 3C, F and I).** No significant differences in BAT radiodensity or cardiometabolic profile (P>0.05) were detected across the different BAT tertiles; these variables are therefore unlikely to influence these comparisons (data not shown).

Figure 3

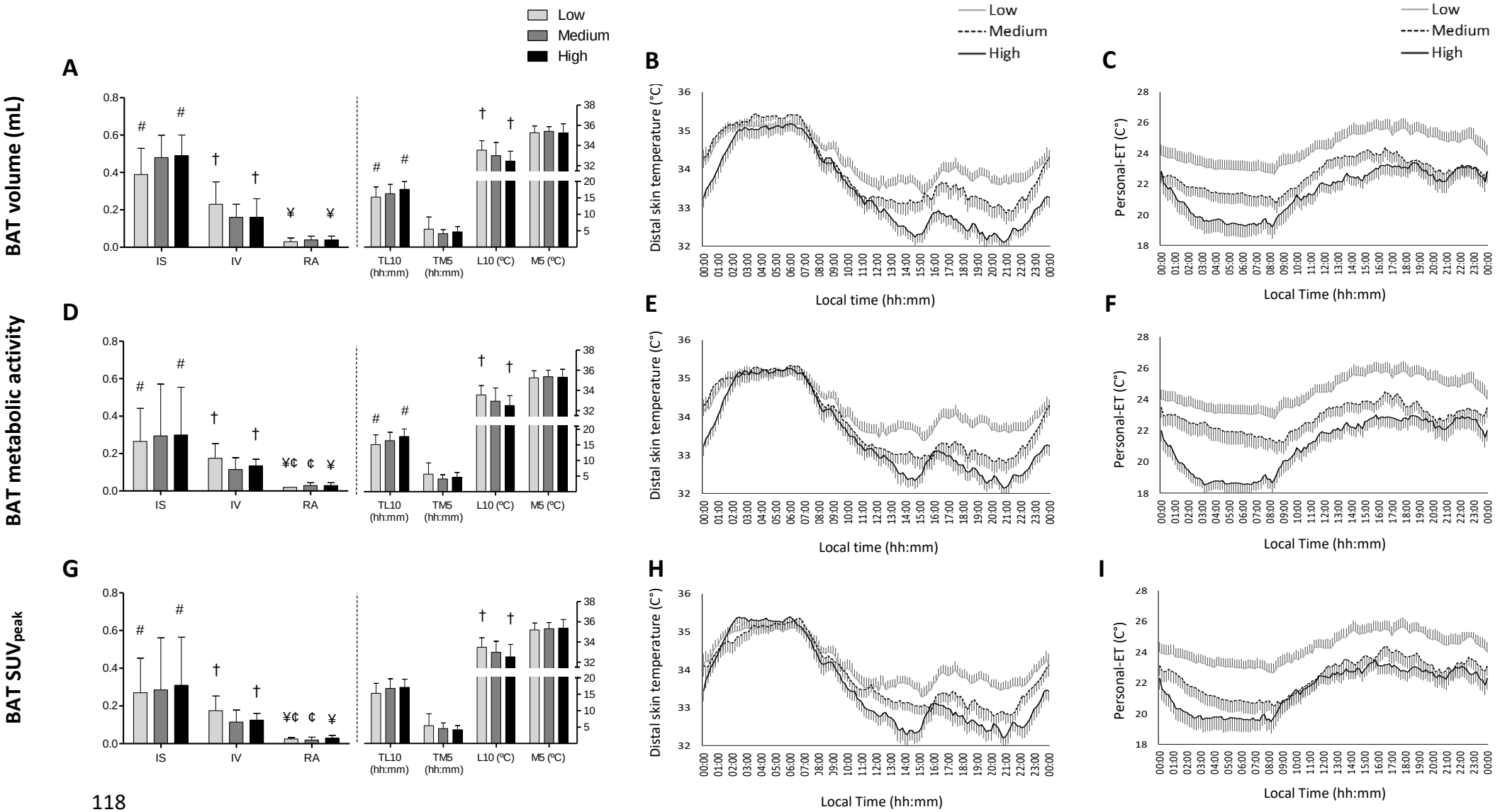
Low
Medium
High
A
B
C
D
E
F
G
H
I
BAT volume (mL)
BAT metabolic activity
BAT SUVpeak
IS
IV
RA
TL10 (hh:mm)
TM5 (hh:mm)
L10 (ºC)
M5 (ºC)
Distal skin temperature (°C)
Distal skin temperature (C°)
Personal-ET (C°)
Local time (hh:mm)
Local Time (hh:mm)

Figure 3. Distal skin temperature (DST) variables and personal environmental temperature (personal-ET) across tertiles for brown adipose tissue (BAT) volume, metabolic activity, and standardized uptake value (SUV) peak (n=77). Mann-Whitney tests (exact significance) with Bonferroni correction (all P≤0.0167) were used to compare DST variables across the tertiles for BAT volume (**Panel A**), metabolic activity (**Panel D**), and SUV_{peak} (**Panel G**). The low, medium and high tertiles comprised a total of 25, 26, and 26 subjects respectively. These tertiles were distributed as: BAT volume (low: 0-27.06 mL, medium: 27.06-104.07 mL, high: >104.07 mL), metabolic activity (low: 0-90.63, medium: 90.63-474.36, high: >474.36 V), and SUV_{peak}: (low: 0-5.38, medium: 5.38-14.1 V, high: >14.1). Symbols show significant differences across tertiles. **Panels B, E** and **H** show the DST mean daily pattern for the tertiles of BAT volume, metabolic activity, and SUV_{peak}, respectively. Similarly, **Panels C, F** and **I** show the personal-ET mean daily patterns for the tertiles of BAT volume, metabolic activity, and SUV_{peak} (n=76). The low, medium and high tertiles comprised a total of 25, 25, and 26 subjects respectively. Data are mean ± standard error. IS: interday stability, IV: intraday variability, L10: mean of the 10 consecutive hours with the lowest values and when they occurred (TL10), M5: mean of the five consecutive hours with the highest values and when they occurred (TM5), RA: relative amplitude.

Association of DST variables with skeletal muscles and descending aorta ^{18}F-FDG uptake

A positive association was detected between IV and the descending aorta SUV_{peak} (P=0.01), even when analyses were adjusted for sex and personal-ET_{L10} (**Table 3**). No other associations were found.

These findings remained unchanged when SUV was expressed with respect to lean body mass (SUV_{LBM}) instead of total body mass (SUV_{BM}) (rendering BAT SUV_{mean} and SUV_{peak}, and skeletal muscle and descending aorta SUV_{peak}; data not shown). No significant association was detected between the time of the day when BAT was assessed and BAT ^{18}F-FDG uptake (performed with all 133 subjects of the present cohort; see Supplementary Material, **Figure S2**).

Table 3. Association of distal skin temperature (DST) variables with skeletal muscle and descending aorta ^{18}F-FDG uptake [mean and peak standardized uptake value (SUV)] in young adults.

	Superficial muscle SUV_{peak}			**Deep muscle SUV_{peak}**			**All muscles SUV_{peak}**			**Descending aorta SUV_{peak}**		
	B	R^2	P	B	R^2	P	B	R^2	P	B	R^2	P
Model 1 (unadjusted)												
IS	0.03	-0.013	0.85	0.27	-0.002	0.37	0.16	-0.004	0.41	-0.56	0.030	0.07
IV	0.001	-0.013	0.99	-0.28	-0.006	0.45	-0.15	-0.008	0.53	1.03	0.080	**0.01**
RA	-0.06	-0.013	0.95	2.06	-0.002	0.35	1.15	-0.005	0.42	-1.48	-0.008	0.52
TL10 (hh:mm)	0.003	-0.011	0.67	0.01	0.005	0.25	0.01	0.005	0.25	-0.003	-0.013	0.84
TM5 (hh:mm)	0.01	-0.003	0.38	-0.004	-0.013	0.83	0.003	-0.013	0.82	-0.03	0.025	0.09
L10 (ºC)	0.002	-0.013	0.9	-0.02	-0.006	0.48	-0.01	-0.009	0.56	0	-0.013	1
M5 (ºC)	0.005	-0.013	0.84	0.02	-0.011	0.65	0.02	-0.010	0.64	-0.07	0.005	0.25
Model 2 (adjusted for sex and personal-ET_{L10})												
IS	-0.066	0.034	0.66	0.02	0.099	0.96	-0.03	0.111	0.89	-0.57	0.095	0.08
IV	0.13	0.038	0.49	0.04	0.099	0.92	0.10	0.113	0.69	1.09	0.136	**0.01**
RA	-1.59	0.056	0.17	-1.78	0.105	0.47	-1.60	0.123	0.31	-2.92	0.072	0.26
TL10 (hh:mm)	-0.002	0.032	0.80	0.005	0.100	0.72	0.002	0.111	0.80	-0.004	0.057	0.79
TM5 (hh:mm)	0.01	0.042	0.37	-0.01	0.101	0.70	0.001	0.111	0.95	-0.03	0.085	0.13
L10 (ºC)	0.02	0.058	0.15	0.03	0.109	0.37	0.03	0.127	0.25	0.04	0.071	0.29
M5 (ºC)	0.01	0.032	0.80	0.03	0.101	0.64	0.01	0.112	0.71	-0.005	0.056	0.94

Linear regression analyses were performed to examine the association of the DST variables with ^{18}F-FDG uptake by the skeletal muscles and the descending aorta. Model 1 was adjusted for no covariate (n=77); Model 2 was adjusted for sex and the mean personal environmental temperature over the L10 period (personal-ET_{L10}, n=76). Non-standardized B coefficient, adjusted R^2 and P values are provided. Significant values are shown in bold (P≤0.05). IS: interday stability, IV: intraday variability, L10: mean of the 10 consecutive hours with the lowest values and when they occurred (TL10), M5: mean of the five consecutive hours with the highest values and when they occurred (TM5), RA: relative amplitude.

Association of DST variables with body composition and cardiometabolic profile

After adjustment for sex and personal-ET_{L10}, IS and RA were inversely related to BMI, LMI, FMI, percentage body fat and visceral adipose tissue (VAT) mass, whereas IV was directly related to these variables (all P≤0.02, **Table 4,** model 2). In addition, L10 was directly associated with BMI, LMI, FMI and VAT mass (all P≤0.01).

Table 4. Association of distal skin temperature (DST) variables with body composition.

	BMI (kg/m^2)			LMI (kg/m^2)			FMI (kg/m^2)			Body fat (%)			VAT mass (g)		
	B	R^2	P	B	R^2	P	B	R^2	P	B	R^2	P	B	R^2	P
Model 1 (unadjusted)															
IS	-13.44	0.118	**0.001**	-6.56	0.119	**0.001**	-6.84	0.071	**0.01**	-7.86	0.008	0.21	-591.33	0.147	**<0.001**
IV	24.58	0.264	**<0.001**	12.22	0.277	**<0.001**	11.83	0.145	**<0.001**	10	0.008	0.20	1024.37	0.290	**<0.001**
RA	-88.43	0.092	**0.004**	-43.11	0.092	**0.004**	-45.63	0.056	**0.02**	-49.63	0.002	0.28	-3713.49	0.103	**0.003**
TL10 (hh:mm)	-0.33	0.027	0.08	-0.20	0.052	**0.02**	-0.12	0	0.32	0.09	-0.012	0.74	-11.14	0.016	0.14
TM5 (hh:mm)	-0.44	0.019	0.12	-0.24	0.029	0.07	-0.17	-0.001	0.34	0.04	-0.013	0.92	-15.8	0.013	0.16
L10 (ºC)	0.67	0.013	0.13	0.17	-0.006	0.45	0.52	0.025	0.09	1.06	0.018	0.13	31.26	0.023	0.10
M5 (ºC)	-1.87	0.064	**0.01**	-1.35	0.157	**<0.001**	-0.46	-0.002	0.36	0.96	-0.004	0.4	-70.02	0.054	**0.02**
Model 2 (adjusted for sex and personal-ET_{L10})															
IS	-12.55	0.214	**0.003**	-3.76	0.506	**0.02**	-8.82	0.098	**0.002**	-17.23	0.183	**0.01**	-518.83	0.275	**0.001**
IV	23.85	0.324	**<0.001**	7.46	0.557	**<0.001**	15.97	0.202	**<0.001**	27.45	0.221	**0.001**	929.34	0.366	**<0.001**
RA	-112.10	0.245	**0.001**	-39.21	0.538	**0.001**	-71.71	0.106	**0.002**	-117.48	0.161	**0.02**	-4398.83	0.292	**<0.001**
TL10 (hh:mm)	-0.24	0.126	0.23	-0.11	0.482	0.12	-0.12	-0.015	0.39	0.005	0.095	0.99	-6.36	0.170	0.4
TM5 (hh:mm)	-0.06	0.109	0.85	0.02	0.465	0.84	-0.07	-0.024	0.75	-0.06	0.095	0.89	-0.18	0.160	0.99
L10 (ºC)	1.27	0.188	**0.01**	0.45	0.507	**0.01**	0.80	0.049	**0.02**	1.25	0.129	0.1	57	0.261	**0.003**
M5 (ºC)	-1.01	0.126	0.23	-0.34	0.473	0.28	-0.69	-0.006	0.24	-1.31	0.108	0.31	-18.52	0.165	0.57

Linear regression analyses were performed to examine the association between DST variables and body composition. Model 1 was adjusted for no covariate (n=77); Model 2 was adjusted for sex and the mean personal environmental temperature over the L10 period (personal-ET_{L10}, n=76). Non-standardized B coefficient, adjusted R^2 and P values are provided. Significant values are shown in bold (P≤0.05). IS: interday stability, IV: intraday variability, L10: mean of the 10 consecutive hours with the lowest values and when they occurred (TL10), M5: mean of the five consecutive hours with the highest values and when they occurred (TM5), RA: relative amplitude. BMI: body mass index, FMI: fat mass index, LMI: lean mass index, VAT: visceral adipose tissue.

After adjusting for sex and personal-ET_{L10}, IS was inversely related (all P≤0.05) to the concentrations of fasting glucose and triglycerides, and to the systolic and diastolic blood pressure. RA was inversely related (all P≤0.05) to the concentrations of fasting glucose, insulin, low-density lipoprotein cholesterol (LDL-C), the HOMA index, and diastolic blood pressure (**Table 5,** Model 2). Further, IS was directly associated with cardiorespiratory fitness, and both, IS and RA were directly associated with high-density lipoprotein cholesterol (all P<0.05). In contrast, increasing IV was related to a poorer cardiometabolic profile, showing a direct association with the concentrations of fasting glucose, insulin, LDL-C, with the HOMA index, and the systolic and diastolic pressure, and an inverse relationship with cardiorespiratory fitness (all P≤0.05, only in **Table 5** Model 2 analysis). It should be noted that a higher L10 was associated with a higher fasting glucose concentration and a higher diastolic blood pressure (all P≤0.05).

Table 5. Association between distal skin temperature (DST) variables and cardiometabolic profile.

	Glucose (mg/dL)		Insulin (µIU/mL)		Total cholesterol (mg/dL)		HDL-C (mg/dL)		LDL-C (mg/dL)		Triglycerides (mg/dL)		HOMA index		C-reactive protein (mg/L)		SBP (mmHg)		DBP (mmHg)		Muscular strength (kg)		CRF (mL/kg/min)	
	B	R^2	B	R^2	B	R^2	B	R^2	B	R^2	B	R^2	B	R^2	B	R^2	B	R^2	B	R^2	B	R^2	B	R^2
Model 1 (unadjusted)																								
IS	**-0.68***	0.046	-1.43	0.031	0.15	-0.013	**2.35*****	0.140	-0.67	-0.010	**-6.02***	0.070	-0.78	0.033	-0.02	-0.013	**-1.34****	0.080	**-1***	0.053	-12.87	0.032	10.89	0.021
IV	**1.27*****	0.118	**2.93****	0.103	1.28	-0.004	**-2.8*****	0.120	2.58	0.021	**8.01****	0.080	**1.64*****	0.120	0.4	-0.005	**2.78*****	0.235	**1.64****	0.097	**27.65*****	0.120	-13.01	0.017
RA	-4.51	0.034	-10.08	0.027	-8.4	-0.002	**13.8****	0.083	-15.43	0.022	**-35.75***	0.040	-5.64	0.032	3.11	0.001	-5.75	0.018	**-10.02****	0.107	-76.24	0.016	72.63	0.011
TL10 (hh:mm)	0.001	-0.013	-0.02	-0.010	-0.01	-0.013	0.052	0.025	-0.03	-0.009	-0.1	-0.002	-0.01	-0.010	-0.02	0.003	-0.03	0.009	-0.003	-0.013	-0.25	-0.004	-0.03	-0.013
TM5 (hh:mm)	-0.002	-0.013	-0.05	0	-0.03	-0.011	0.065	0.014	-0.04	-0.010	-0.15	-0.001	-0.03	0	-0.03	0.008	**-0.07***	0.050	-0.02	-0.009	-0.74	0.020	-0.11	-0.013
L10 (ºC)	0.04	0.006	0.09	0	0.14	0.001	-0.097	0.007	0.22	0.019	0.35	0.009	0.05	0.001	-0.05	0.001	-0.002	-0.014	0.13	0.073	-0.23	-0.012	0.02	-1.360
M5 (ºC)	-0.07	0.005	-0.18	0.009	0.06	-0.013	**0.32***	0.072	-0.02	-0.013	-0.56	0.009	0.16	0.011	-0.01	-0.013	**-0.29****	0.103	-0.07	-0.004	**-3.95*****	0.120	-0.65	-0.010
Model 2 (adjusted for sex and personal-ET_{L10})																								
IS	**-0.78***	0.095	-1.45	0.056	-0.6	-0.012	**1.56***	0.267	-1.24	-0.022	**-5.39***	0.052	-0.79	0.068	-0.02	-0.031	**-1.12***	0.289	**-1.02***	0.024	-2.81	0.580	**15.61***	0.041
IV	**1.42*****	0.159	**2.88****	0.110	2.8	0.020	-1.22	0.226	**3.99***	0.032	6.55	0.041	**1.62****	0.136	0.37	-0.026	**2.39*****	0.376	**1.78****	0.064	9.24	0.590	**-21.91***	0.048
RA	**-6.99****	0.117	**-13.47***	0.073	-17.28	0.023	**10.08***	0.247	**-25.23***	0.040	-33.76	0.028	**-7.62***	0.092	3.85	-0.014	-7.21	0.269	**-11.75****	0.095	-69.89	0.598	108.96	0.023
TL10 (hh:mm)	0.01	0.031	0.02	0.016	-0.01	-0.014	0.012	0.206	-0.04	-0.028	0.02	-0.011	0.01	0.026	-0.02	-0.022	-0.01	0.233	0.02	-0.030	-0.05	0.579	-0.11	-0.017
TM5 (hh:mm)	0.03	0.051	0.05	0.022	-0.01	-0.014	-0.012	0.205	-0.02	-0.032	0.08	-0.009	0.03	0.033	-0.02	-0.027	-0.03	0.237	0.02	-0.035	-0.1	0.579	-0.25	-0.016
L10 (ºC)	**0.08***	0.085	0.15	0.045	0.2	0.008	-0.122	0.232	0.31	0.019	0.38	0.011	0.08	0.060	-0.05	-0.018	0.07	0.245	**0.15****	0.060	1.07	0.599	-1.42	0.014
M5 (ºC)	-0.05	0.030	-0.14	0.021	-0.14	-0.011	0.073	0.208	-0.14	-0.029	-0.35	-0.005	-0.08	0.031	0.01	-0.031	-0.14	0.251	-0.07	-0.031	0.29	0.579	0.25	-0.018

Linear regression analyses were performed to examine the association between DST variables, and the glycemic and lipid markers, the HOMA index, C-reactive protein (n=76), systolic and diastolic blood pressure (n=75), muscular strength (n=77) and cardiorespiratory fitness (n=76). Model 1 was adjusted for no covariate; Model 2 was adjusted for sex and the mean personal environmental temperature over the L10 period (personal-ET_{L10}). In Model 2, one subject's data was missing for all the variables. The non-standardized B coefficient and adjusted R^2 values are provided. All variables related to the cardiometabolic profile, except for muscular and cardiorespiratory fitness, were square root-transformed before analysis given their non-normal distribution. Values in bold with asterisks indicate significance differences *P≤0.05, **P≤0.01, ***P≤0.001. CRF: cardiorespiratory fitness, DBP: diastolic blood pressure, HDL: high density lipoprotein cholesterol, HOMA: homeostatic model assessment of insulin resistance, IS: interday stability, IV: intraday variability, LDL-C: low density lipoprotein cholesterol, L10: mean of the 10 consecutive hours with the lowest values and when they occurred (TL10), M5: mean of the five consecutive hours with the highest values and when they occurred (TM5), RA: relative amplitude, SBP: systolic blood pressure.

DISCUSSION

The present results show that a more flattened, fragmented, and less stable DST daily rhythm is associated with a lower BAT ^{18}F-FDG uptake, although the variance these differences explain is relatively small (~5-14%). However, these associations became non-significant after adjusting for sex and the habitual temperature to which subjects were exposed during the week that DST was measured. Taken together, these findings suggest that the relationship between the functioning of the circadian system (indirectly measured by the daily rhythm of DST) and BAT variables is masked by environmental and behavioural factors under free-living conditions. Importantly, the participants exposed to a colder personal-ET had a lower DST throughout the day, and had a 3-5 times greater BAT volume and activity (high BAT tertile) compared to those who were exposed to a warmer personal-ET (low BAT tertile). A more flattened, fragmented and less stable DST daily rhythm was also associated with a higher BMI and body fat accumulation, and a poorer cardiometabolic profile, suggesting that chronodisruption may result in adverse health effects.

Despite the growing evidence suggesting the importance of proper circadian rhythmicity in the preservation of metabolic homeostasis, in humans, the interaction between the biological clock and the metabolic activity of BAT has only recently been addressed. Lee et al. (2016)[26] reported that the thermogenic response of the BAT (measured by supraclavicular skin temperature profiling) was coupled with glycemic excursions in young healthy adults. Via *in vivo* and *in vitro* experiments, these authors were able to show that this circadian coupling was accompanied by changes in the expression of UCP1, GLUT4, and Rev-erb alpha. However, it should be noted that the supraclavicular skin temperature is only a proxy of BAT activity[45,46] - and that it is influenced by body composition[47]. Indeed, the supraclavicular fossa is surrounded by blood vessels, skeletal muscles and lymph nodes (among others)[48]. Consequently, changes in the supraclavicular skin temperature are more likely to be a response to blood perfusion than to BAT thermogenic activity. Clearly, more studies are needed to clarify the relationship between the biological clock and BAT function in humans. In line with Lee et al. (2016)[26], the present results initially suggested a link between DST daily rhythm and BAT ^{18}F-FDG uptake. In fact, those subjects who had a lower quality DST daily rhythm also had a lower ^{18}F-FDG uptake (although only a small part of BAT

^{18}F-FDG uptake variance was explained by DST variables). This would seem coherent since subjects with a disrupted circadian system (i.e., lower IS and RA, higher fragmentation, phase-advance or delay) - a consequence of their social habits - would be more likely to have altered behavioural and physiological responses[3], which might extrapolate to BAT function.

However, when the analyses were adjusted for sex and personal-ET_{L10} or evaluation wave, the previous associations disappeared. The observed relationship between the DST daily rhythm and BAT volume and activity was therefore greatly influenced by the effect of the ambient temperature to which the participants were exposed. This is probably explained in that both physiological responses are sympathetically driven. The fact that this relationship is masked by environmental factors suggests that when searching for strategies to optimize BAT function and its potential health benefits, simply modifying human behaviour (e.g., exposure to cold and modifying clothing) would be more efficient than designing strategies to exploit diurnal fluctuations (if they exist) in BAT activity. Accordingly, the subjects who were exposed to a lower personal-ET, both during the day and night, were those with the highest BAT volume, metabolic activity and SUV_{peak} (high BAT tertile); indeed, they were 3-5 times those of the subjects in the low BAT tertile (see Figure 3C, F and I, and Table S1). In line with the present results, previous studies have shown that the day when the ^{18}F-FDG-PET/CT scan is performed is related to BAT volume and activity[49], and that environmental temperature and personal-ET are inversely related to these BAT measures[31,36,50]. A better understanding of the mechanisms underlying the relationship between chronic exposure to cold and BAT function is needed. For instance, beyond the effect of seasonality, it might be possible that the subjects in the present low BAT tertile had a low tolerance to cold exposure, and therefore would expose themselves to a higher personal-ET, or use more strongly insulating clothing, i.e., relying more on their behaviour for thermoregulation than on endogenous forms of heat production such as BAT thermogenesis. It may also be that the subjects with a DST daily rhythm of lower quality relied more on vasomotor responses, again suggesting that they are more behaviourally responsive and do not rely so much on thermogenesis. Future studies are warranted to examine the factors that influence

chronic exposure to low temperatures and induce a higher reliance on thermogenic processes.

A temperature increase during the night phase was recorded, concordant with the reduction in core body temperature recorded at sleep onset (see Figs. 1 and 3)[51–54]. Earlier research in adult humans showed an increase in DST during the night to be associated with shortened sleep latency as well as an increase in sleep time and depth[51,55], demonstrating a link between the thermoregulatory and sleep centers[56–58]. Interestingly, the TM5 (an indicator of midsleep timepoint and sleep onset) of the present subjects showed a weak inverse association with BAT SUV_{mean}, even after adjustment for sex, personal-ET_{L10} and body composition variables (data not shown). However, when analyses were performed to see whether the midsleep timepoint or chronotype of the subjects (assessed using the Munich Chronotype Questionnaire; see Supplementary Material) were related to the BAT volume, SUV_{mean}, and SUV_{peak}, no association was found. In addition, DST showed an initial reduction followed by an increase during the postprandial state, which may be indicative of peripheral vasoconstriction and vasodilation (respectively) and the redistribution of blood flow to ensure proper nutrient digestion, absorption and distribution[59]. There was also a reduction in the DST after the participants woke up, in a manner phase-opposed with the increase in core temperature and morning heat production peak[52,59].

Current knowledge supports the hypothesis that the disruption of the circadian system is associated with obesity and altered metabolism, probably explained by the desynchronization of different organ- or pathway-specific circadian rhythms[3,60]. Indeed, it has been shown that a disrupted DST daily rhythm is a predictor of a lower weight-loss effectiveness[61] and that it is related to certain features of metabolic syndrome[62]. Accordingly, the present results show that a more flattened, fragmented and less stable DST daily rhythm is associated with a higher BMI, greater body fat accumulation (all $P \leq 0.03$, Table 4), and a poorer cardiometabolic profile (see Table 5) - even after adjusting for sex and personal-ET. It is also noteworthy that greater stability and lesser fragmentation showed an association with greater cardiorespiratory fitness (after adjustment for sex and personal-ET_{L10}), a major predictor of cardiovascular health[63]. The fact that a reduction in L10 was related to lower fasting glucose concentrations and a lower diastolic blood pressure, reinforces the idea that chronic

exposure to colder temperatures might have health benefits (reducing the risk of type 2 diabetes and cardiovascular disease). The only previous experiment examining the relationship of DST daily patterns with obesity and cardiometabolic risk factors[62] did not examine whether their results were independent of the personal-ET to which participants were exposed, generating a potential bias.

The skeletal muscles may also play a key role in non-shivering thermogenesis[64,65] via the interaction between sarcolipin and the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCAs) enzymes. This may be especially important in large mammals (such as humans), in which BAT content is small in relation to body size. In the present work, no relationship was seen between the DST rhythm and skeletal muscle ^{18}F-FDG uptake during cold exposure, but it should be remembered that this may not be the best way to indirectly assess non-shivering thermogenesis, which is influenced by muscle tension and shivering thermogenesis. Further, mild cold exposure is likely to predominantly promote the oxidative metabolism of fat[66].

The present work suffers an inherent limitation of studies with a cross-sectional design: no causal relationships can be discerned. In addition, for logistic reasons, no control over how participants dressed during the DST measurements was enforced, and some of the associations found were weak, and may have been influenced by the error generated by multiple comparisons. Further, given the highly radioactive nature of ^{18}F-FDG PET/CT scans, BAT rhythmicity was not assessed (only one measurement was made). It might therefore be argued that the present results are influenced by the cross-subject temporal variation in BAT ^{18}F-FDG uptake. However, when it was examined whether BAT ^{18}F-FDG uptake measures were related to the time of the day when they were made, no relationship was seen (**see Figure S2**). Despite being the most extensively used technique for measuring BAT activity, ^{18}F-FDG-PET-CT scanning also has some limitations that might prevent cold-induced BAT metabolic activity from being accurately estimated; the method provides a measure of glucose metabolism while fatty acids are the main substrate for human brown adipocytes[9]. Whether the present findings will be reproduced when other radiotracers such as ^{15}O-oxygen, ^{11}C-acetate or ^{18}F-fluoro-6-thia-heptadecanoic acid are used to quantify BAT metabolism remains to be seen. Further studies investigating the relationship between BAT

function and the circadian rhythm of a wider range of physiological markers not sympathetically driven or conditioned by the environmental temperature, are warranted. Moreover, studies under well-controlled laboratory conditions, such as those using misalignment protocols (e.g., recurring non-24 h behavioural cycle), are needed to examine the relative contribution of the endogenous circadian cycle (e.g., DST daily rhythm) to BAT metabolic activity, independent of behavioural factors.

CONCLUSION

The possible relationship between circadian functioning (indirectly measured via the daily rhythm of DST) and BAT ^{18}F-FDG uptake is masked by environmental and likely behavioural factors. The subjects exposed to the lowest personal-ET had the lowest DST throughout the day, and had BAT volume and activity values 3-5 times those of subjects exposed to higher personal-ETs. Further studies examining which factors influence persons to seek chronic exposure to lower temperatures, and thus rely more strongly on thermogenic processes, are warranted.

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SUPPLEMENTARY MATERIAL

Sociodemographic and clinical data

A self-reported survey was used to collect sociodemographic (i.e., age, professional status, educational level) and clinical (i.e., medical problems or issues that might condition the inclusion of the subjects in the study, history of cardiovascular risk markers or disease in subject's family) data. All subjects were provided with continuous instructions on how to complete the survey.

Cardiometabolic profile

Glycemic and lipids markers, HOMA index and C-reactive protein

Serum glucose, total cholesterol, high density lipoprotein-cholesterol and triglycerides were assessed following standard methods using an AU5832 automated analyzer (Beckman Coulter Inc., Brea CA, USA). Low density lipoprotein-cholesterol was estimated as: [total cholesterol – HDL-C – (triglycerides/5)], with all units in mg/dL[1]. Serum insulin was measured using the Access Ultrasensitive Insulin chemiluminescent immunoassay kit (Beckman Coulter Inc., Brea CA, USA). The HOMA index was calculated as *(insulin (μU/mL) x glucose (mmol/L)/22.5*[2]. C-reactive protein was measured by immunoturbidimetric assay, employing the same automated analyzer as above.

Systolic and diastolic blood pressure

An Omron M6 upper arm blood pressure monitor (Omron Healthcare Europe B.V. Hoofddorp, The Netherlands) was used to determine the systolic and diastolic blood pressure, with subjects seated and relaxed. Measurements were taken at three time points, and the mean determined for use in later analyses.

Muscular strength

Handgrip strength was assessed as a proxy of muscular strength on a different day to when the cooling experiments and PET/CT scan were performed. Briefly, handgrip strength was determined using an adjustable grip TKK 5101 Grip - D hand dynamometer (Takei, Tokyo Japan). Subjects were asked to squeeze gradually and continuously for a few seconds, and were encouraged to do their best when

performing the tests. All tests were performed using the optimal grip-span[3]. Each subject performed two attempts with each hand, with the arm fully extended and maintaining the trunk erect. The maximum score for each hand was recorded in kilograms and the mean score of the left and right hand used in analyses.

Cardiorespiratory fitness

Subjects' maximum oxygen consumption ($VO_{2\ max}$) was determined via a maximum exercise test using a Pulsar treadmill (H/P/Cosmos Sport & Medical GMBH, Nußdorf, Germany), based on the modified Balke [protocol4]. O_2 consumption and CO_2 production were measured by indirect calorimetry (CPX Ultima CardiO_2, Medical Graphics Corp, St Paul, USA) using a Model 7400 oronasal mask (Hans Rudolph Inc., Kansas City, MO, USA) equipped with a Prevent™ metabolic flow sensor (Medgraphics Corp., St. Paul, MN, USA). The criteria for achieving $VO_{2\ max}$ were: a respiratory exchange ratio ≥1.1, a plateau in VO_2 (change of <100 mL/min in the last three consecutive 60 s stages), and a heart rate within 10 beats/min of the age-predicted maximum (208–0.7 x age)[5]. When no plateau in VO_2 was reached, $VO_{2\ peak}$ was measured.

Prevalence of metabolic syndrome

Specific cardiometabolic risk factors were recorded and the prevalence of metabolic syndrome then determined based on two classifications: i) the National Cholesterol Education Program Adult Treatment Panel III (ATP III) criteria[6], and ii) the International Diabetes Federation criteria (IDF)[7]. For (i), subjects were deemed to have metabolic syndrome when they had three or more of the following risk factors: waist circumference (WC) ≥102 cm for men and 88 cm for women; triglycerides ≥150 mg/dL; HDL-cholesterol <40 mg/dL for men and <50 mg/dL for women; systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg; plasma glucose >110 mg/dL. For (ii subjects were deemed to have metabolic syndrome when they had central obesity plus at least two of the following risk factors: waist circumference (WC) ≥94 cm for men and ≥80 cm for women; triglycerides ≥150 mg/dL; HDL-cholesterol <40 mg/dL for men and <50 mg/dL for women; systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg; plasma glucose ≥100 mg/dL.

Physical activity level

The DST daily rhythm can be masked by several factors, including physical activity[8]. This is partially explained by the fact that those people who perform exercise or physical activity generally have a higher average skin temperature during exercise. This could lead to variations in the DST and its daily rhythm. To avoid any confounding effect, physical activity was objectively measured using a wrist-worn GT3X+ accelerometer (ActiGraph, Pensacola, FL, US) for 7 consecutive days (24 h/day)[9], and adjusted for when examining the association between the DST daily rhythm and BAT ^{18}F-FDG uptake. The subjects were given detailed information on how to wear the accelerometer. Once the recording was finished and the raw data processed and analyzed (described extensively in Acosta et al. [2018][10]), an overall indicator of physical activity (mG) during time awake was established using the ENMO (Euclidean Norm Minus One) metric.

Chronotype

Alignment between the biological and social clocks is important for the management of obesity[11]. Indeed, the central circadian clock, which harmonizes all the processes ranging from cellular to whole-body physiology with environmental cues, is often influenced by social obligations (for instance, humans align their sleep and wake times to their work schedule or social events). To quantitatively characterize these individual differences in daily schedule, several variables related to subject chronotype were recorded: i) midsleep timepoint (the midpoint between sleep onset and waking up); ii) chronotype (the midsleep timepoint corrected for the sleep deficit on free days); and iii) social jetlag (the difference between midsleep timepoints on free days and on workdays). All these variables were calculated using the Munich Chronotype Questionnaire, employing the formula proposed by Roennerberg et al. (2012). Sleep onset was recorded for workdays (Sunday to Thursday) and free days (Friday and Saturday), and the sleep offset for workdays (Monday to Friday) and weekends (Saturday and Sunday), from the sleep diaries the subjects completed over the days they wore the DST sensor.

Table S1. Distal skin temperature (DST), personal environmental temperature (personal-ET; see Methods and Materials), and ^{18}F-fluorodeoxyglucose (^{18}F-FDG) PET/CT scan variables, by evaluation wave.

	Wave 1 (n=18)*		**Wave 2 (n=21)**		**Wave 3 (n=18)**		**Wave 4 (n=20)**		**P-value**
DST variables									
IS	0.38	(0.13)	0.42	(0.14)	0.50	(0.09)	0.51	(0.11)	**0.004**
IV	0.23	(0.11)	0.22	(0.10)	0.12	(0.05)	0.15	(0.10)	**0.002**
RA	0.02	(0.01)	0.03	(0.01)	0.04	(0.02)	0.04	(0.02)	**<0.001**
TL10 (hh:mm)	15:26	(03:43)	15:13	(02:33)	18:01	(02:09)	17:04	(02:15)	**0.005**
TM5 (hh:mm)	04:35	(03:07)	03:49	(00:50)	05:04	(01:43)	04:25	(01:33)	0.25
L10 (ºC)	33.91	(0.66)	33.37	(0.70)	32.34	(1.14)	32.34	(1.21)	**<0.001**
M5 (ºC)	35.36	(0.60)	35.21	(0.60)	35.38	(0.69)	35.29	(0.92)	0.88
Personal-ET variables									
IS	0.30	(0.10)	0.31	(0.11)	0.31	(0.14)	0.27	(0.12)	0.63
IV	0.27	(0.09)	0.22	(0.10)	0.23	(0.10)	0.25	(0.11)	0.47
RA	0.05	(0.02)	0.07	(0.03)	0.12	(0.05)	0.09	(0.04)	**<0.001**
TL10 (hh:mm)	06:07	(01:54)	05:36	(01:40)	06:52	(04:16)	07:45	(04:51)	0.23
TM5 (hh:mm)	16:32	(02:37)	16:43	(02:39)	18:19	(03:58)	19:02	(05:14)	0.12
L10 (ºC)	24.83	(1.21)	22.25	(1.14)	18.53	(2.16)	19.15	(2.95)	**<0.001**
M5 (ºC)	27.42	(1.24)	25.71	(1.78)	23.51	(3.08)	23.10	(4.09)	**<0.001**
^{18}F-FDG PET/CT variables									
BAT radiodensity (HU)	-61.86	(16.78)	-56.14	(10.21)	-58.63	(9.72)	-58.39	(10.52)	0.55
BAT volume (mL)	15.08	(29.16)	61.42	(48.29)	104.12	(50.64)	95.89	(69.70)	**<0.001**
BAT SUV_{mean}	2.02	(1.45)	3.60	(1.64)	4.80	(1.78)	4.37	(1.59)	**<0.001**
BAT SUV_{peak}	3.55	(3.85)	9.81	(6.90)	17.14	(9.37)	13.64	(6.88)	**<0.001**
Superficial muscle SUV_{peak}	0.56	(0.11)	0.60	(0.15)	0.66	(0.22)	0.61	(0.09)	0.23
Deep muscle SUV_{peak}	0.89	(0.18)	1.14	(0.38)	1.25	(0.38)	1.18	(0.25)	**0.005**
All muscle SUV_{peak}	0.72	(0.14)	0.86	(0.25)	0.94	(0.26)	0.89	(0.13)	**0.01**
Descending aorta SUV_{peak}	1.48	(0.26)	1.70	(0.46)	1.59	(0.33)	1.62	(0.29)	0.29

Values are means (standard deviation). *For the group of subjects assessed in evaluation wave 1, a few measurements of personal-ET and BAT radiodensity were missing (remaining n=17 and 16 respectively). One-way ANOVA was used to detect any difference among evaluation waves. Significant values (P<0.05) are shown in bold. IV: intraday variability, L10: mean of the 10 consecutive hours with the lowest values and when they occurred (TL10), M5: mean of the five consecutive hours with the highest values and when they occurred (TM5), PET/CT: positron emission tomography combined with computed tomography, RA: relative amplitude.

Table S2. Association between distal skin temperature (DST) variables and brown adipose tissue (BAT) volume and standardized uptake values (SUV mean and peak), after adjusting for potential confounders (n=76).

	BAT volume (mL)			**BAT SUV_{mean}**			**BAT SUV_{peak}**		
	B	R^2	P	B	R^2	P	B	R^2	P
Model 1 (adjusted for sex, personal-ET_{L10}, and body mass index)									
IS	83.64	0.307	0.12	0.44	0.228	0.80	4.20	0.259	0.58
IV	-85.12	0.295	0.27	0.77	0.228	0.76	-8.92	0.263	0.41
RA	648.61	0.305	0.13	8.94	0.232	0.52	74.17	0.272	0.22
TL10 (hh:mm)	4.84	0.326	**0.04**	0.07	0.236	0.37	0.43	0.274	0.19
TM5 (hh:mm)	-1.53	0.285	0.67	-0.23	0.272	**0.04**	-0.78	0.282	0.12
L10 (ºC)	-9.33	0.306	0.13	-0.09	0.230	0.64	-1.18	0.275	0.17
M5 (ºC)	-0.90	0.283	0.93	0.07	0.228	0.82	-0.48	0.257	0.73
Model 2 (adjusted for sex, personal-ET_{L10}, and lean mass index)									
IS	62.77	0.280	0.24	-0.15	0.264	0.93	1.36	0.284	0.85
IV	-42.61	0.269	0.57	2.02	0.272	0.39	-2.22	0.284	0.83
RA	489.33	0.279	0.26	1.72	0.264	0.90	41.80	0.289	0.48
TL10 (hh:mm)	4.70	0.306	**0.05**	0.05	0.269	0.51	0.35	0.295	0.28
TM5 (hh:mm)	-1.70	0.268	0.64	-0.22	0.306	**0.04**	-0.75	0.308	0.12
L10 (ºC)	-7.69	0.282	0.22	-0.02	0.264	0.93	-0.83	0.293	0.33
M5 (ºC)	-2.10	0.267	0.84	0.02	0.264	0.94	-0.71	0.286	0.61
Model 3 (adjusted for sex, personal-ET_{L10}, and fat mass index)									
IS	85.44	0.310	0.11	0.92	0.226	0.60	6.03	0.263	0.43
IV	-83.39	0.297	0.27	-0.27	0.223	0.91	-12.52	0.270	0.24
RA	620.56	0.306	0.14	12.94	0.232	0.35	87.77	0.278	0.14
TL10 (hh:mm)	4.6	0.326	**0.04**	0.07	0.234	0.32	0.45	0.276	0.17
TM5 (hh:mm)	-1.44	0.287	0.68	-0.23	0.267	**0.04**	-0.77	0.281	0.12
L10 (ºC)	-9.02	0.307	0.14	-0.14	0.228	0.49	-1.32	0280	0.12
M5 (ºC)	-0.84	0.285	0.93	0.11	0.224	0.74	-0.35	0.257	0.80
Model 4 (adjusted for sex, personal-ET_{L10}, and body fat percentage)									
IS	85.64	0.321	0.10	1.48	0.245	0.38	8.18	0.284	0.27
IV	-72.55	0.305	0.31	-1.20	0.240	0.61	-14.97	0.294	0.14
RA	561.37	0.314	0.17	15.94	0.253	0.23	96.76	0.301	0.09
TL10 (hh:mm)	4.25	0.329	0.06	0.07	0.248	0.32	0.43	0.291	0.17
TM5 (hh:mm)	-1.54	0.297	0.66	-0.23	0.280	**0.04**	-0.76	0.296	0.12
L10 (ºC)	-8.36	0.315	0.16	-0.17	0.246	0.38	-1.42	0.301	0.09
M5 (ºC)	-0.73	0.295	0.94	0.15	0.240	0.64	-0.19	0.272	0.89

Linear regressions were performed to examine the association between DST variables and BAT volume, SUV_{mean}, and SUV_{peak}, after adjusting for sex, mean personal environmental temperature over the L10 period (personal-ET_{L10}), and body mass index (Model 1); for sex, personal-ET_{L10}, and lean mass index (Model 2); for sex, personal-ET_{L10}, and fat mass index (Model 3), and for sex, personal-ET_{L10}, and body fat percentage (Model 4). Non-standardized B coefficient, adjusted R^2 and P values are provided. Significant values are shown in bold (P≤0.05). IS: interday stability, IV: intraday variability, L10: mean of the 10 consecutive hours with the lowest values and when they occurred (TL10), M5: mean of the five consecutive hours with the highest values and when they occurred (TM5), RA: relative amplitude.

Figure S1

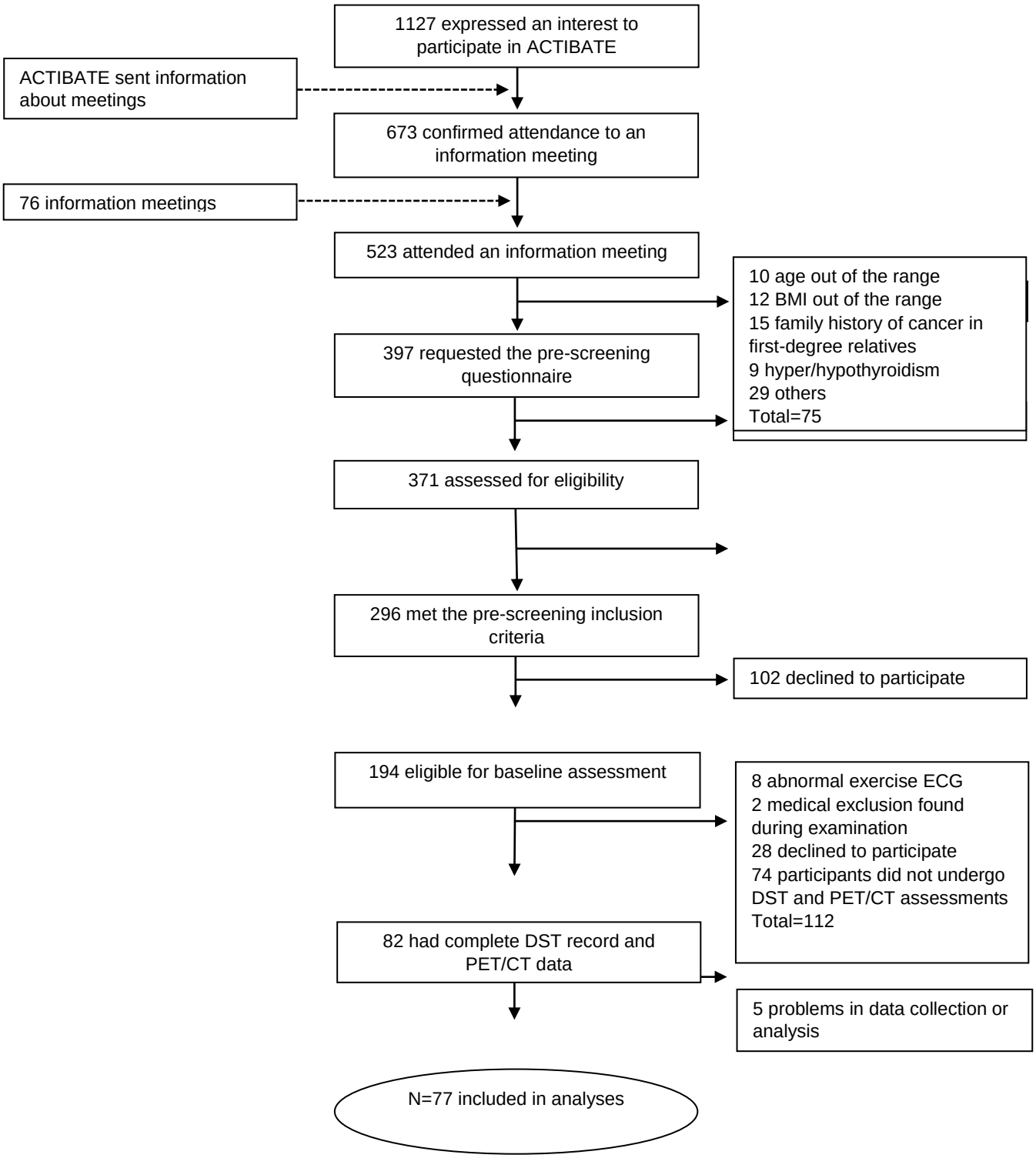

Figure S1. Enrolment flow-chart. BMI: body mass index, ECG: electrocardiogram, PET/CT: positron emission tomography combined with computed tomography, DST: distal skin temperature.

Figure S2

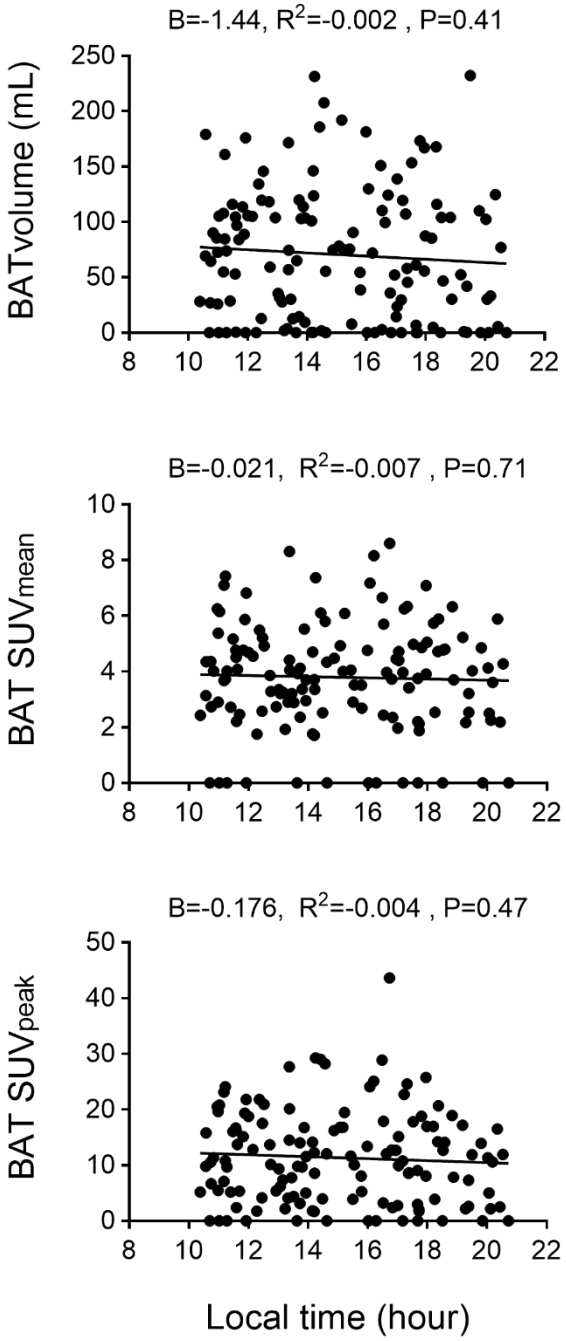

Figure S2. Association between brown adipose tissue (BAT) ^{18}F-fluordeoxyglucose uptake and the time of the day when it was assessed in young sedentary adults. Simple linear regressions were perfumed to examine the association between BAT volume (**Panel A)**, the mean standardized uptake value (SUV_{mean}, **Panel B**) and SUV_{peak} **(Panel C)**, with the time of the day when BAT was assessed. Non-standardized beta coefficient, adjusted R^2 and P values are provided. These analyses were performed with data from the complete study cohort of the ACTIBATE study (n=133, 88 women, 22±2 years old, body mass index: 24.9±4.8 kg/m^2).

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STUDY III

Diurnal variations in cold-induced thermogenesis and brown adipose tissue in young adults

ABSTRACT

Increasing cold-induced thermogenesis (CIT) has been proposed as a potential strategy to counteract positive energy balance and face obesity at long-term. However, it remains unknown whether CIT follows a diurnal fluctuation, being susceptible of being maximized at specific moments of the day in humans.

In this study, we performed a within subject-crossover experiment (Study 1, n=17, 7 women), to examine whether CIT has diurnal variations. Subjects attended to the lab during 2 different days (separated by 72h, random order), one day during the morning (9:00 am), and another day during the evening (19:00 pm). During these days, subjects underwent a mild cold exposure, and CIT was assessed for 60 minutes using indirect calorimetry. In an independent cross-sectional study (Study 2, n=133, 88 women), we additionally examined whether brown adipose tissue (BAT) ^{18}F-fluordeoxygelucose uptake and radiodensity after cold stimulation, were related to the time of the day when they were assessed. As a part of the Study 2, we also examined whether CIT was associated with the time of the day when it was assessed in a sub-cohort of participants (n=41, 27 women). Subjects came to the lab 48-72 hours after BAT assessment (at a similar schedule – 8:00 to 18:00h), underwent a personalized cold exposure, and CIT was assessed for 60 minutes using indirect calorimetry.

In the Study 1, no significant effect of the moment of the day was observed on CIT, neither on its kinetics during the mild cold exposure (all P>0.05). In the Study 2, CIT and BAT ^{18}F-fluordeoxyglucose uptake and radiodensity were not associated with the time of the day at which subjects were assessed (all P>0.05). Therefore, our results suggest that CIT and the BAT variables examined in this work might not have diurnal variations. Further research is needed to corroborate our findings.

INTRODUCTION

In simple terms, body weight is determined by the balance between total energy expenditure (TEE) and energy intake in humans. TEE is a multicomponent construct, explaining resting energy expenditure (REE) the major part of it (60-70% of REE), together with other components such as physical activity energy expenditure and adaptive thermogenesis (meal and cold-induced thermogenesis)[1]. Theoretically, and if not compensated by any compensatory mechanism (e.g., food intake), changes in any of the previous components could lead to changes in body weight, and therefore become potential strategies to prevent or treat obesity and related comorbidities.

Humans are endothermic beings, which means that they are able to modulate their heat production to compensate for heat dissipation, maintaining a stable core temperature (i.e., set point) against environmental challenges. In cold temperatures, and when changes in the behaviour and skin blood flow are not able to fully compensate heat loss, energy expenditure increases, an adaptive physiological response known as cold-induced thermogenesis (CIT)[2]. It has been shown that in small mammals (i.e., mice), CIT can increase energy expenditure up to 60% above REE under normal animal housing temperatures (~22-23ºC), and that when exposed at an ambient temperature of 4ºC, energy expenditure can be increased up to 4-5 times[3,4]. This clearly shows the substantial energetic demands of thermoregulatory effectors, and suggests that CIT might be used as a potential strategy to induce a negative energy balance. In humans, CIT normally ranges from 15 to 30% above REE for its non-shivering component[5,6], being able to increase up to 5-fold above REE when shivering is maximum[7]. Nevertheless, humans present a much lower area surface to volume ratio than small mammals, and have a better capacity to regulate their behaviour, therefore, relying less on CIT to maintain a stable core temperature[2]. In addition, human CIT presents a high-inter-individual variability[6,8]. Hence, it still remains unknown how we can safely and comfortably harness CIT in humans in the fight against obesity[6,9].

Importantly, most physiological responses in our organism are under the control of our circadian system[10,11], a network of hierarchically organized structures that regulate the body´s temporal organization in relation to the environment [12–15].

Human reports have shown that heat production (i.e., REE) fluctuates throughout the day under unmasking and forced desynchrony protocols[10,16], together with changes in heat loss in order to regulate the core body temperature. Similarly, the circadian system plays a dominating role in the morning/evening difference in diet induced thermogenesis (DIT)[17–19], which seems to be independent of the behavioural cycle influence. Nevertheless, to our knowledge, there no studies regarding whether CIT follows a diurnal/circadian rhythmicity in humans. To know whether CIT varies at different circadian phases (e.g., morning vs. evening), and whether harnessing it at specific time frames could help us to maximize its effects on the daily TEE, would be of great interest.

Furthermore, whether CIT would follow diurnal fluctuations, it is still unknown which of its components is contributing to these fluctuations. Brown adipose tissue (BAT), a thermogenic tissue with a unique ability to uncouple mitochondrial respiration through UCP1 protein, is the major contributor to CIT during mild cold exposure in rodents[20]. Interestingly, the metabolic activity and formation of BAT has been shown to be tightly under the regulation of the biological clock in mice[21–26]. Whether this applies to humans is still unknown.

Therefore, in the present paper we aimed to: i) examine whether CIT has diurnal variations, using a within-subject cross over study design, ii) to examine in an independent cross-sectional study, whether BAT ^{18}F-FDG uptake is different across specific moments of the day, and whether it is related to the time of the day when it was assessed.

MATERIALS AND METHODS

Research design and participants

A total of 150 young healthy adults from 2 independent cohorts participated in the present studies. Among them, 17 participants (7 women, 25±3 years old) were enrolled in the study 1 (see Table 1). In addition, a total of 133 participants (82 women, 22 ±2 years old) were part of the ACTIBATE study (Study 2), a randomized controlled trial aiming to examine the effect of exercise on BAT volume and activity [ClinicalTrials.gov, ID: NCT02365129 [27]]. The participants were normally recruited through advertisements in the electronic media and leaflets. Inclusion criteria were to

be sedentary (participants reported to practice <20 min of moderate-vigorous physical activity on <3 days/week), not to smoke or take any medication, to have an unfluctuating body weight in the last 3 months (changes <3 kg), not to present any cardiometabolic disease (i.e. hypertension, diabetes, etc.), not to be pregnant, and not to have any family history of cancer. All assessments from the Study 1 were performed during the month of December 2017 and January 2018, whereas the assessments from the Study 2 were performed during the months of October, November, and December 2015 and 2016 (in 8 temporal waves - 4 per year), in Granada (south of Spain). The study protocols and the written informed consents were performed in accordance with the Declaration of Helsinki (revision of 2013). The studies were approved by the Human Research Ethics Committee of the University of Granada (nº 924), and by the Human Research Ethics Committee of the Junta de Andalucía (nº 0838-N-2017).

Procedures

Study 1. This was a within-subject cross over study designed to examine whether CIT has diurnal variations. With this purpose, subjects attended to the lab during 2 different days, one day during the morning (approximately at 9:00 am), and another day during the evening (around 19:00 pm), during which REE and CIT were assessed. The order of these study days was randomly selected, and experimental procedures were similar in both days (except for the time scheduling).

Briefly, subjects came to the research centre, and confirmed having met the pre-study conditions: i) to arrive in fasting state (10 hours), ii) to sleep as usual, iii) to refrain from any moderate or vigorous physical activity (within 24 and 48 hours, respectively), and iv) not to consume any alcoholic or stimulant beverages (within 6 hours) or drugs which could have affected the peripheral circulation (within 24 hours). Then, they voided their bladders, dressed-up with the same standardized clothes (clo: 0.20) and moved into a warm (22-23ºC) quiet room. Before the evaluation, the participants lay down on a reclined bed, in supine position covered by a sheet for 20 minutes. Later, REE was assessed using indirect calorimetry for 30 minutes following the current methodological recommendations[28]. Subjects were instructed to breathe normally, and not to talk, fidget, or sleep. After assessing REE, subjects were moved into a cold room (19.5-20ºC), and lay down on a bed with the same reclined position as the one used for the REE assessment. Subjects were dressed with a temperature-controlled

water perfused cooling vest, whose temperature was set 4ºC above their shivering threshold. The shivering threshold was defined as the individualized lowest tolerable temperature at which shivering was not reported by subjects, or observed by the evaluators, during a gradual cooling protocol performed 48h before CIT experiments[29]. Then, the CIT measurement was performed during two consecutive 30-minute periods, separated by a 5-minute pause to recalibrate the metabolic cart, during which they continued exposed to cold. Of note, when required, the temperature of the cooling vests water was increased in order to avoid shivering during CIT experiments. Subjects were continually reminded to breathe normally, and not to talk, fidget, or sleep.

Study 2. In this independent cross-sectional study, we examined whether BAT ^{18}F-FDG uptake was different across specific moments of the day, and whether it was related to the time of the day when they it was assessed.

Subjects came to the lab normally between 8:00 am and 18:00 pm, and confirmed that they had met the same pre-study conditions than in the study 1. Subjects dressed with standardized cloths, voided their bladders, and stayed in a warm room (22-23ºC) during 30 minutes. Afterwards, subjects entered a cold room (19.5-20°C), where they sat down and wore a water circulation cooling vest (Polar Products Inc., Ohio, USA) set ~4°C above their shivering threshold, for 60 minutes in order to induce BAT activation. The shivering threshold was calculated as previously, in a gradual cooling protocol performed 48-72 hours before. After 60 minutes of personalized cold exposure we injected a bolus of ^{18}F-FDG (183.52±12.21 MBq = 2.7 MBq/kg) and raised the water temperature of the cooling vest ~1°C for the last 60 minutes in order to avoid shivering. After 2 hours of personalized cold exposure, subjects went into the positron-emission tomography combined with computed tomography (PET/CT) scan to quantify BAT ^{18}F-FDG uptake and radiodensity. A low dose CT (120kV, 11mA) for attenuation correction and anatomic localization was performed directly prior to each PET scan. Then, we performed a static PET consisting of 2 bed scans (6 min each one), performed from the *atlas vertebrae* to *thoracic vertebrae 6*[29]*.* The personalised cold exposure and the ^{18}F-FDG-PET/CT acquisition were performed according to the current methodological recommendations[30].

^{18}F-FDG-PET/CT analysis

PET/CT scans were analysed using the software based on Beth Israel plugin for FIJI http://sourceforge.net/projects/bifijiplugins/. We calculated the standardized uptake value (SUV) as [^{18}F-FDG uptake (kBq/ml) / (injected dose [kBq] / patient weight [g])]. To determine BAT ^{18}F-FDG uptake and radiodensity, we outlined 6 regions of interest (ROIs), from *atlas vertebrae* to *thoracic vertebrae* 4, using a 3D-axial technique[31]. These ROIs comprised the supraclavicular, laterocervical, paravertebral and mediastinal regions. Within these ROIs, a SUV threshold for a voxel to be considered BAT was computed as SUV ≥ [1.2 / (lean body mass/body mass)], and a fixed range of Hounsfield units (HU, -190 to -10) was applied (following the current recommendations)[30]. We obtained BAT volume, SUV_{mean} and SUV_{peak}, as well as BAT radiodensity (used as a control)[32,33]. We additionally outlined 1 single slice-ROI to determine the SUV_{peak} in the descending aorta (reference tissue). For confirmatory analyses, we computed BAT SUV_{mean} and SUV_{peak} as a product of % lean body mass (SUV_{LBM})[31].

Estimation of energy expenditure and nutrient oxidation rates

The indirect calorimetry measurements for both REE and CIT were performed using 2 different metabolic carts (for logistical reasons), a CCM Express (CCM), or a Ultima CardiO2 (MGU) device (Medgraphics Cardiorespiratory Diagnostic, Saint-Paul, USA), which were equipped with a directconnect™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA)[34,35]. A neoprene facemask was hooked up to the metabolic cart. The flow calibration was performed by a 3-L calibration syringe at the beginning of every test day, and the gas analyzers were calibrated using 2 standard gas concentrations before every 30-minute bout of indirect calorimetry measurement following the manufacturers' instructions. For each participant, we always used the same metabolic cart for REE and CIT.

Indirect calorimetry data were averaged every minute and downloaded from the Breeze Suite (8.1.0.54 SP7) software. For REE, we selected the most stable 5-minute period [i.e., the one with the lowest average coefficients of variance (CV) of oxygen consumption, carbon dioxide production, minute ventilation, and respiratory exchange ratio)], after excluding the first 5 minutes recorded[34]. To be considered as a stable period, the CV of the above variables had to be <10%, except for the respiratory

exchange ratio - CV<5%. To obtain a single representative value of CIT, we divided the 60 minutes recorded into 4 periods (i.e. 15 minutes each). We then selected the most stable 5-minute period within every 15-minute period. Finally, we used the 4 selected 5-minute periods together with the REE to calculate the area under the curve (trapezoidal rule), expressing it as a percentage of REE[8] [AUC (%REE)]. The same procedure was applied to obtain a single representative value of nutrient oxidation rates during cold exposure.

Oxygen consumption and carbon dioxide production for each selected data point were used to estimate energy expenditure, and carbohydrates and fat oxidation (CHO_{ox} and FAT_{ox}, respectively). Energy expenditure was estimated through Weir´s abbreviated equation[36]. For CHO_{ox} and FAT_{ox} estimations, we used Frayn´s equations[37]. We included urinary nitrogen data into the equations for nutrient oxidation estimations.

Body composition

We measured the participants´ body composition by dual-energy x-ray absorptiometry scan (Discovery Wi, Hologic, Inc., Bedford, MA, USA), obtaining the absolute values of lean and fat mass, as well as the fat mas percentage. Weight and height were measured by a Seca scale and a stadiometer (model 799, Electronic Column Scale, Hamburg, Germany).

Statistical analyses

Descriptive statistics for continuous and categorical variables were used to show the characteristics of the study participants. We performed a one-way ANOVA to compare energy expenditure and nutrient oxidation rates during resting and cold conditions across different moments of the day (morning vs. evening, Study 1). In addition, linear mixed model analyses were used to examine the kinetics of energy expenditure and nutrient oxidation rates during the cold exposure in the morning vs. the evening.

We used one-way analysis of variance (ANOVA) to examine whether there was any significant difference in BAT ^{18F}-FDG uptake and radiodensity across 3 different moments of the day (tertiles of the time at which the PET/CT scan was performed): morning, afternoon, and evening. Single linear regression analyses were also performed to examine whether BAT variables were related to the time of the day when the PET-CT scan was performed. Later analyses were performed to examine whether results from the previous analyses remained similar after adjusting for

potential confounders (determined by exploratory correlation analyses). The level of significance was set at $P<0.05$. The statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS version 24, Inc. Chicago, IL, USA).

RESULTS

Table 1 shows the descriptive characteristics of the participants by study. A total of 17 (n= 8 for nutrient oxidation related variables) and 133 subjects were respectively included in the Study 1 and 2.

Table 1. Characteristics of study participants

	Study 1 (within-subject crossover design)	**Study** 2 (cross-sectional design)
	Diurnal CIT variation	BAT ^{18}F-FDG uptake across different moments of the day
	(n=17)	(n=133)
Women (%)	7 (41)	88 (66)
Age (years)	25 (3)	22 (2)
BMI (kg/m^2)	23.1 (2.8)	24.9 (4.8)
Lean mass (kg)	44.6 (11.3)	42.2 (10)
Fat mass (kg)	17.3 (4.5)	25.1 (9.2)
Fat mass (%)	27.3 (6.4)	35.9 (7.4)
REE (kcal/day)	1492 (249)	

Data are presented as mean (standard deviation) and number (percentage) for continuous and categorical variables (respectively). *For the Study 1, REE (resting energy expenditure) was computed as the mean of the REE in the morning and the evening. BAT: brown adipose tissue, BMI: Body mass index.

Diurnal variations of CIT

Figure 1 shows the REE and CIT, as well as the resting and cold-induced (CI) nutrient oxidation rates during 2 different moments of the day: morning (09:00h) vs evening (19:00). No significant differences were found when comparing REE and resting CHO_{ox} in the morning vs. the evening (all P>0.05, **Panels A and B**). However, we observed that resting FAT_{ox} values were higher in the evening than in the morning (mean±standard deviation, P-value: 0.053±0.013 vs. 0.027±0.017, P=0.004, Panel C). No significant differences (all P>0.05) were found when we examined the change in CIT, and CI CHO_{ox} and FAT_{ox}, expressed as the AUC (%REE), in the morning vs. the evening (**Panels D, F and E**).

We additionally examined the kinetics of energy expenditure and CHO_{ox} and FAT_{ox} throughout the cold exposure protocol. Energy expenditure steeply increased during the initial moments of cold exposure (until minute 20-25), and continued to slightly increase until the end of the cold exposure (effect of cold exposure P<0.001, **Panel G**). There was no significant effect of the time of the day or interaction between the effects of the time of the day and cold exposure on CIT (all P≥0.757). Regarding the kinetics of CHO_{ox} and FAT_{ox} during the cold exposure, they were respectively decreased and increased until approximately half of the cold exposure (minute 30), in which a shift phase happened (**Panels H and I**). No significant effect of the time of the day or interaction between the effects of the time of the day and cold exposure was found on CHO_{ox} and FAT_{ox} (all P≥0.26).

Of note is also that for few variables (i.e., CI CHO_{ox} and CI FAT_{ox}) in which parametric assumptions were not totally met, we additionally performed non-parametric tests, and all the results remained similar. Furthermore, some participants reported shivering – or it was visually detected by the evaluators - during the cold exposure, in both, or in one of the days (despite the fact the cold stress to which each participant was submitted was similar during both days). Hence, as sensitivity analyses, we examined whether the results remained similar after excluding those participants. Overall, results replicated and no differences of the CIT and CI CHO_{ox} and FAT_{ox} (neither in its kinetics) were observed in the morning vs. evening (all P>0.05).

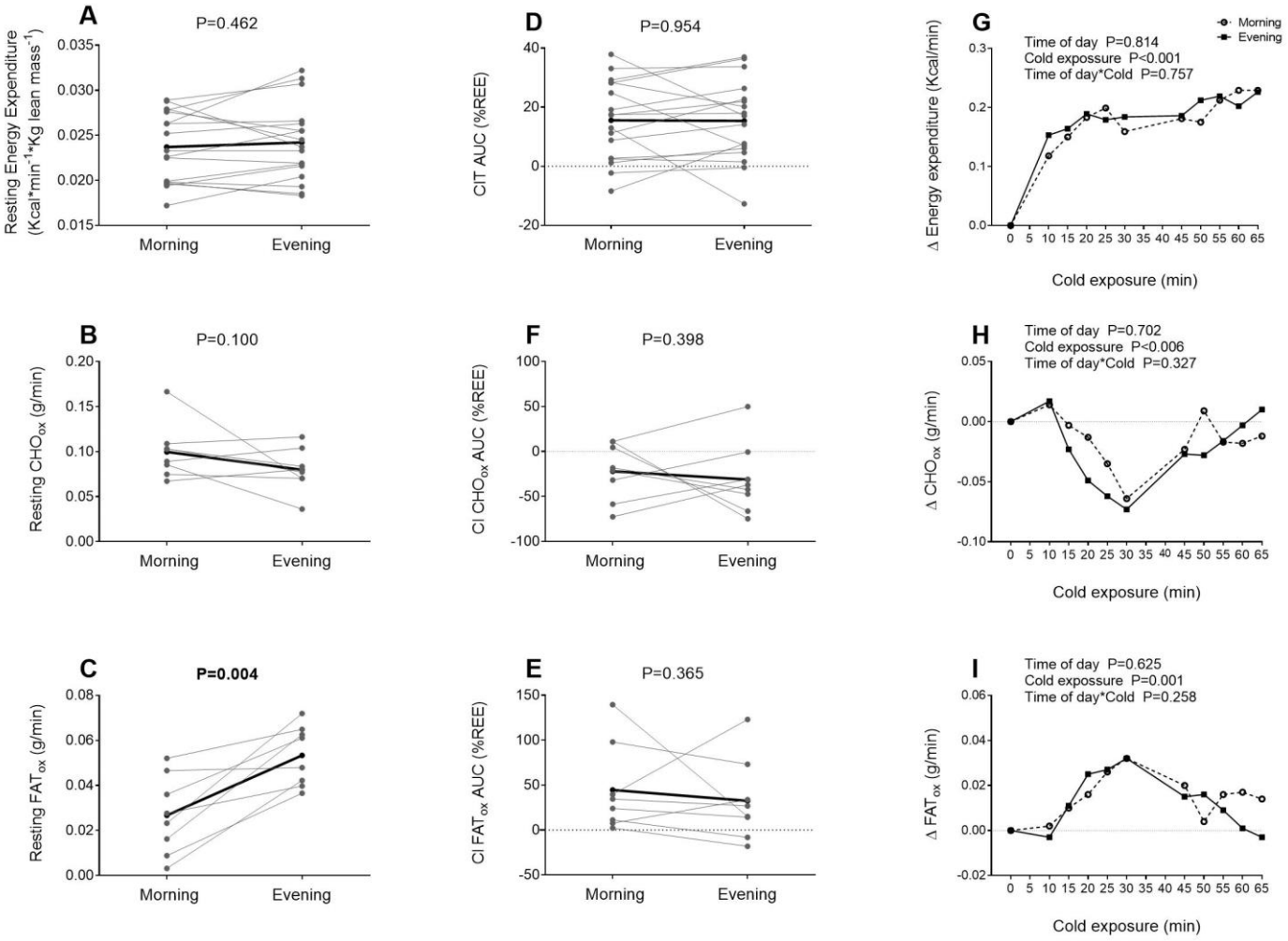

Figure 1. Diurnal variations in cold-induced thermogenesis (CIT) in young adults. Study 1 (within-subject cross over study). **Panels A, B and C** show the resting energy expenditure (REE) and carbohydrate and fat oxidation (CHO_{ox} and FAT_{ox}) in 2 different moments of the day (morning, 09:00h vs. evening, 19:00h). **Panels D, E and F** show the CIT as well as the cold induced (CI) CHO_{ox} and FAT_{ox} in the morning vs the evening. The black bar in the above Panels show the mean change in the corresponding variable when comparing the morning vs. the evening. **Panels G, H and I** show the kinetics of CIT and CI CHO_{ox} and FAT_{ox} during the cold exposure, in the morning vs. the evening. The sample size for REE and CIT was 17 participants, whereas the sample size for nutrient oxidation related variables was of 8 participants. Paired sample t-test were used to compare all measures between the morning vs. the evening. A linear-mixed model analysis was employed to examine the kinetics of CIT and CI CHO_{ox} and FAT_{ox} during the morning vs the evening.

BAT ^{18}F-FDG uptake across different moments of the day

No significant differences in BAT ^{18}F-FDG uptake (volume, SUV_{mean}, SUV_{peak}) and radiodensity were found across different moments of the day (morning, afternoon, and evening, all P≥0.3) (**Figure 2, Panels A-D**). No changes were either observed when the above variables were compared across quintiles instead of tertiles of the time when the PET/CT scan was performed (data not shown).

Similarly, when we examined the relationship of BAT ^{18}F-FDG uptake related variables and BAT radiodensity with the time of their assessment (**Figure 2, Panels E-H**), we did not find any significant association (all P≥0.33). These results remained similar when: i) adjusted for sex, body composition variables, the HOMA index, or the date when the PET/CT scan was performed; ii) the analyses were run excluding those participants who were PET- (those who showed a BAT volume, SUV_{mean} and SUV_{peak} =0, n=120); iii) BAT SUV_{mean} and SUV_{peak} were computed as a product of % lean body mass (SUV_{LBM}) (data not shown). The descending aorta ^{18}F-FDG uptake (SUV_{peak}) neither showed a significant relationship with its time of assessment (P=0.55, Supplementary Material, **Figure S1**).

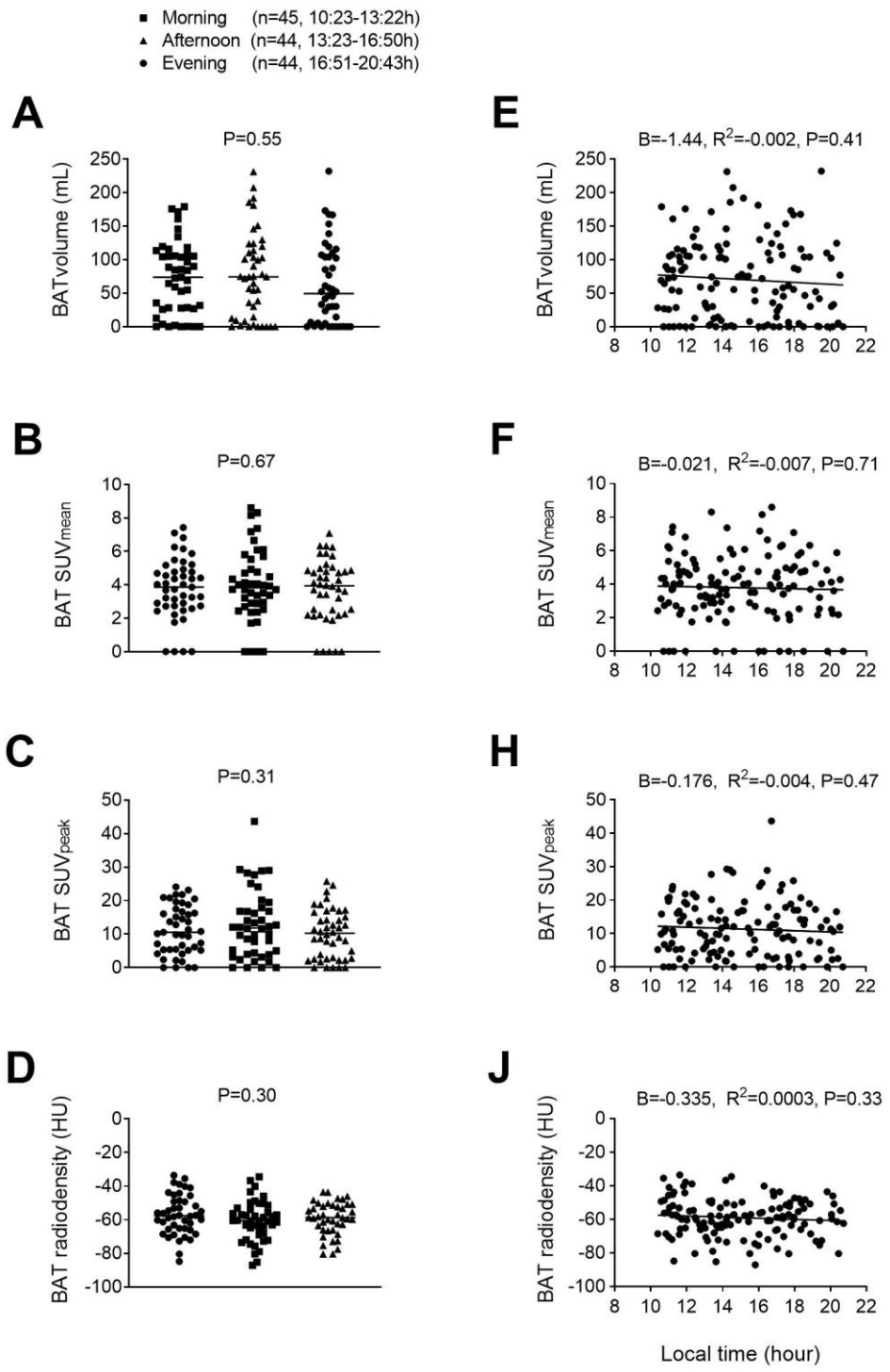

Figure 2. Brown adipose tissue (BAT) ^{18}F-fluordeoxyglucose uptake across different moments of the day (n=133). Study 2 (cross-sectional design). Analyses of variance (ANOVA) were performed to compare BAT volume (**Panel A),** mean standardized uptake value (SUV_{mean}, **Panel B**) peak standardized uptake (SUV_{peak}, **Panel C)**, and BAT radiodensity **(Panel D),** across different time groups (morning, afternoon, and evening). P-value for the between-subject effect is provided. Categorization within each group (morning, afternoon or evening) was performed based on the time at which the PET/CT scan started (1st tertile=morning group, 2nd tertile=afternoon group, 3rd tertile=evening group). Single linear regressions were performed to examine the association of the above variables with the time of the day when BAT was assessed **(Panels E-H)**. Non-standardized beta coefficient, adjusted R square, and P-value are provided.

DISCUSSION

The results from the present study show that there were no differences in CIT and CI CHO_{ox} and FAT_{ox}, neither in their kinetics during a mild cold exposure, across 2 different moments of the day (i.e., morning and evening). Similarly, no differences in the energy expenditure, neither in CHO_{ox} during resting conditions were observed. Only resting FAT_{ox} seemed to be higher in the evening than in the morning. Furthermore, BAT ^{18}F-FDG uptake was similar across different moments of the day. Taken together, these findings suggest that REE, CIT and BAT ^{18}F-FDG uptake may not exhibit diurnal variations in young healthy adults under these protocols.

Our results showing that CIT was similar in the morning and evening may seem counterintuitive, since previous reports have shown that under thermoneutral conditions, REE and nutrients oxidation rate vary with a circadian phase - being REE and FAT_{ox} higher during the biological evening[10]. These oscillations happen together with body temperature changes to regulate the set point[11], independently of the sleep-wake and activity-related effects[10]. In addition, the circadian system plays a dominating role in the morning/evening difference in diet induced thermogenesis (DIT)[17–19], which shares common underlying mechanisms with CIT[38,39] - the sympathetic system is the main effector of both. Furthermore, several areas of the hypothalamus involved in the integration of afferent signals and control of effector responses related to cold, are also involved in the circadian control of body temperature and energy expenditure (e.g., the dorsomedial nucleus)[2,40]. These facts together made us hypothesizing that the time of the day would have an effect on energy expenditure and nutrient oxidation rates during cold exposure, although the opposite was observed.

To date, no previous studies have examined whether CIT exhibits a diurnal/circadian rhythmicity in humans, precluding any comparison with our results. Nevertheless, our results appear not to concur with those obtained from rodent models. Experiments carried out in Golden hamsters[41] have reported that CIT was greater during the low phase of core body temperature rhythm (i.e., inactive phase). Another study in rats[42] suggested that there was a time-of-day dependent modulation of energy expenditure and substrates oxidation during cold exposure (0-5ºC). Tokizawa

et al. (2009)[43] also found that fasted mice exposed to cold (20ºC) increased their heat production in the dark phase and maintained body core temperature, whereas in the light phase, heat production was less, resulting in hypothermia. However, this was not observed in fasted control mice exposed to thermoneutral conditions. Interestingly, such differences in thermoregulatory responses were attenuated in Clock mutant mice[43]. All these studies together may indicate that there is an interaction between the biological clock and thermoregulatory system (including CIT) during cold exposure in rodents. Nevertheless, the locomotor activity of these rodents was not controlled, and food and water were provided ad libitum in most studies, factors which are likely to have masking effects on CIT (e.g., a peak of heat production could be related to physical activity energy expenditure or DIT rather than CIT). Beyond the methodological differences, differences between our results and those reported in rodents are likely to be explained by the vast differences between species in terms of their morphology and physiology[44].

Additionally, BAT ^{18}F-FDG uptake and radiodensity (used as a control) were not different across different moments of the day, neither were related to the time of the day when they were assessed. This seems to be contrary to previous evidence from rodent models [21–23], showing that: i) the formation and metabolic function of BAT are under the circadian clock regulation; ii) BAT transcriptome possesses a robust degree of rhythmicity [24,25]; iii) several key nuclear receptors in circadian rhythmicity, such as Rev-erb alpha and PER2, play a fundamental role in the modulation of UCP1 expression and BAT thermogenesis[23,26]; iv) BAT receives the input from several nucleus and zones of the hypothalamus, shaping the rhythms of systemic glucose and lipids as well as of body temperature and energy expenditure[2,21,45–47]. Despite the accumulative evidence suggesting that the circadian rhythm of body temperature and metabolism are tightly attached to a proper BAT function in rodents[48], evidence in humans is insufficient. Until now, only 1 study[49] has suggested that glucose utilization by human BAT is coupled with its heat production (indirectly measured with wireless thermometers) in a circadian manner. Nevertheless, this study has important caveats that may question the previous conclusion[50]. Hence, whether BAT thermogenesis follows a diurnal/circadian rhythmicity is still under debate, although given its low contribution to daily energy expenditure under mild cold conditions (10±5 kcal/day) in humans[51], to

harness it at specific time frames might not have clinical implications/health benefits. Further research is warranted on this field.

All results together suggest that CIT and the BAT variables measured might not have diurnal variations, shedding some light on the still unexplored relationship between these outcomes and the biological clock. Nevertheless, it is important to consider that CIT was assessed at only 2 different temporal moments of the day (at 9:00 and 19:00). The inclusion of more measurements throughout the day would have allowed us to obtain more accurate results (i.e., higher resolution). Moreover, these results are likely to be influenced by the protocol/design that we applied. For instance, for the assessment of CIT we used a within-subject crossover design (study 1) trying to standardize pre-experimental conditions. However, there are several masking factors (e.g., sleep-wake cycle, type and timing of the meals of the previous days, outdoors temperature to which participants were exposed before the experiment, etc.) which were not controlled, and might have hampered us from discerning any difference in CIT and CI CHO_{ox} and FAT_{ox} in the morning compared to the evening. Future studies should aim to perform constant routine or forced desynchrony protocols to examine the diurnal rhythmicity of CIT under unmasking conditions, or to examine the endogenous component of CIT, independently of the behavioural cycles (respectively). In the case that CIT would show a diurnal/circadian rhythmicity when applying these protocols, it remains to be seen whether harnessing CIT at specific times of the day would have implications on daily energy expenditure and body weight regulation at long term. Previous reports have shown that exposure to an acute cold protocol after 10 to 31 days of cold acclimation (using cold-air or liquid-perfused cooling suits[52–55], increases CIT to a similar extent than the values observed before the cold acclimation; however, these studies failed to find any change in weight or body composition[52,53]. This suggests that the increase in daily energy expenditure induced by CIT may be compensated by adaptive mechanisms (e.g., increase in food intake, decrease in physical activity, increased metabolic efficiency etc.), and therefore, calls into question whether exposure to cold at certain times of the day would be an efficient strategy to combat obesity. Of note also, that for logistical reasons we used 2 different metabolic carts (always the same for each participant). However, sensitivity analyses showed that the type of metabolic cart used did not have any effect on CIT.

Regarding the assessment and quantification of BAT ^{18}F-FDG uptake, we used the most extended technique and updated recommendations for this purpose[30], although it is necessary to consider that a static ^{18}F-FDG PET-CT scan has several limitations that might not allow for the accurate estimation of cold-induced BAT metabolic activity[56]. Whether the present findings will be replicated when using other radiotracers such as ^{15}O-oxygen, ^{11}C-acetate or ^{18}F-fluoro-6-thia-heptadecanoic acid (FTHA) to quantify BAT metabolism, remains to be seen. Future studies should examine how continuously measured BAT activity varies along the day, and how it relates to the oscillation of other physiological functions. This fact will be confined to the advance of the current technologies to assess BAT metabolic activity in a non-invasive and non-ionizing manner, or by the validation of indirect markers that accurately reflect its activity. Finally, to mention is that these results cannot be generalized to a population beyond ours (young healthy adults).

In conclusion, our findings suggest that CIT and the BAT variables measured might not have diurnal variations in young healthy adults. This information is valuable, and shed some light on the unexplored relationship between these variables and the biological clock, although further research is needed to corroborate our findings.

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SUPPLEMENTARY MATERIAL

Figure S1

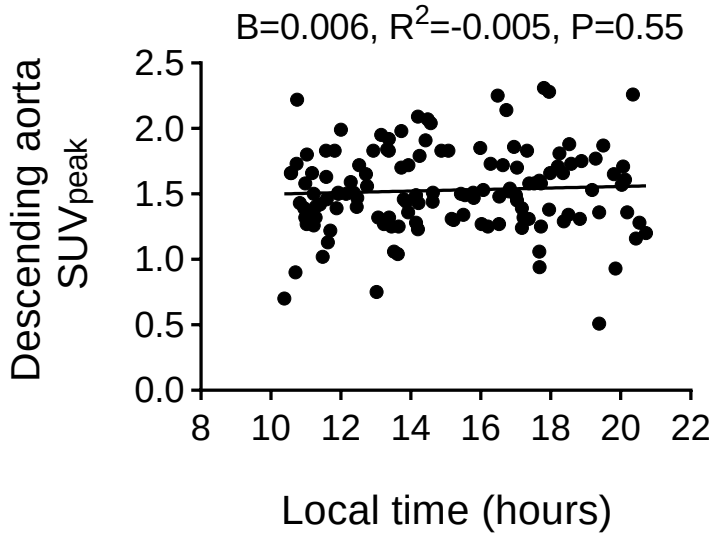

Figure S1. Association of the descending aorta peak standardized uptake value (SUV_{peak}) with the time of the day when it was assessed in young adults (n=133). Single linear regression analysis was performed.

STUDY IV

Sleep duration and quality are not associated with brown adipose tissue volume or activity – as determined by ^{18}F-FDG uptake, in young, sedentary adults

ABSTRACT

Short sleep duration and sleep disturbances have been related to obesity and metabolic disruption. However, the behavioural and physiological mechanisms linking sleep and alterations in energy balance and metabolism are incompletely understood. In rodents, sleep regulation is closely related to appropriate brown adipose tissue (BAT) thermogenic activity, but whether the same is true in humans has remained unknown. The present work examines whether sleep duration and quality are related to BAT volume and activity (measured by ^{18}F-FDG) and BAT radiodensity in humans.

A total of 118 healthy adults (69% women, 21.9±2.2 years, BMI: 24.9±4.7 kg/m^2) participated in this cross-sectional study. Sleep duration and other sleep variables were measured using a wrist-worn accelerometer for 7 consecutive days for 24 h/day. The Pittsburgh Sleep Quality Index was used to assess sleep quality. All participants then underwent a personalized cold exposure to determine their BAT volume, activity and radiodensity (a proxy of the intracellular triglyceride content), using static positron emission tomography combined with computed tomography scan.

Our result showed that neither sleep duration nor quality were associated with BAT volume nor activity (the latter represented by the mean and peak standardized ^{18}F-FDG uptake values), nor radiodensity (all P>0.1). The lack of association remained after adjusting the analyses for sex, date of PET/CT, and body composition.

While experiments in rodent models indicate a strong relationship to exist between sleep regulation and BAT function, it seems that sleep duration and quality may not be directly related to the BAT variables examined in the present work.

INTRODUCTION

Sleep is an active, regulated metabolic state essential for health[1,2]. An extensive body of epidemiological and experimental evidence has shown that sleep curtailment and disturbances are related to an increased risk of obesity and the disruption of metabolic and endocrine functions[2–4], becoming a new avenue for intervention. However, the behavioural and physiological mechanisms linking sleep and alterations in energy balance and metabolism are not well understood.

Brown adipose tissue (BAT) is a specialized thermogenic organ that dissipates heat, especially during cold exposure, a process mediated by uncoupling protein 1 (UCP1)[5]. In rodents, BAT is characterized by its strong thermogenic capacity, but also by its contribution to metabolic homeostasis via the uptake of nutrients[5,6] and its role as an endocrine organ[7]. Until a decade ago it was thought present only in small rodents and neonates, but a number of studies simultaneously confirmed its existence and metabolic activity in adult humans[8–11]. Since its "rediscovery", manipulating human BAT activity has been contemplated as means of combating obesity and diabetes, although recent evidence calls into question whether its impact on energy balance is as substantial as initially thought[6,12,13].

The systems that regulate energy balance and metabolic homeostasis are often linked to the neural circuits that modulate sleep duration and quality[14]. For instance, the dorsomedial nucleus in the hypothalamus, which projects into different nuclei and areas related to energy expenditure, feeding, and sleep regulation[15–18], plays a key role in BAT sympathetic premotor neuron excitement. It is also well known that the sleep and thermoregulatory mechanisms are closely related[19–22]. In adult humans, an increase in the distal skin temperature during the night (which is phased-opposed to the decrease in core temperature)[23], is associated with shortened sleep latency and increases in sleep duration and depth[24,25]. Therefore, it is biologically plausible that sleep regulation and BAT thermogenic function are related in humans.

The first observations of this potential relationship arose from experiments in sleep-deprived rodents; these animals showed a hyperphagic response and an increase in energy expenditure despite a falling body temperature[26,27]. This led to the hypothesis that BAT is activated to compensate for the heat loss typically observed in sleep

deprivation states[27]. Accordingly, Balzano et al.[26] confirmed the 5´-deiodinase activity of BAT to be prompted when rats were sleep-deprived. Later experiments[28,29] in rodents showed that intact BAT thermogenesis is required for restorative sleep responses after induced sleep loss, and that BAT has an important function as a sleep-inducing signalling organ. In fact, sleep deprivation induces a 6-fold increase in UCP-1 mRNA expression in the BAT of wild-type mice[28]. Interestingly, the activation of BAT is related to rodent rapid and non-rapid eye movement (REM and NREM respectively) sleep phases under normal and inflammatory conditions[28,30–32].

There is, therefore, evidence that supports the idea of crosstalk between sleep regulation and BAT function - at least in these animals.

Whether these observations also apply to healthy humans remains to be seen. The present study examines whether sleep duration and quality are related to BAT volume and activity (both determined via ^{18}F-FDG uptake) and radiodensity (a proxy of the intracellular triglyceride content)[33] after personalized cold exposure in a cohort of young healthy adults. Unfortunately, nearly all the cross-sectional and longitudinal studies[1–4,14,34–36] that have examined the evidence for a relationship between sleep curtailment/other sleep variables and obesity have suffered from: 1) the lack of an objective assessment of these variables, ii) not simultaneously assessing sleep duration and quality, and iii) only including BMI among the measured body composition variables. A complementary aim of this work was therefore to determine whether sleep duration and quality are associated with obesity and body composition.

METHODS

Research design and participants

A total of 137 young healthy adults took part in this cross-sectional study; all were recruited into the ACTIBATE study[37] (ClinicalTrials.gov, ID: NCT02365129) via advertisements in electronic media and leaflets. Figure S1 shows a flow-chart explaining how they were enrolled in the present work. The inclusion criteria were: age 18-25 years old, having a sedentary lifestyle (i.e., undertaking <20 min moderate-vigorous physical activity <3 days/week at baseline), to not be a smoker or take any medication, having had a stable body weight over the last 3 months (changes <3 kg), to have no cardiometabolic disease (e.g., hypertension or diabetes), and to have no first-

degree relative history of cancer. Positron emission tomography combined with computed tomography (PET/CT) assessments were completed over eight dates distributed over October, November and December of 2015 and 2016 (four per year); all assessments were made in Granada (south of Spain). The study was approved by the University of Granada Ethics Committee on Human Research (nº 924) and by that of the *Servicio Andaluz de Salud*. All work was performed in accordance with the Declaration of Helsinki (2013 revision); all subjects gave their written informed consent to be included.

Procedures

Sleep duration and quality

Sleep duration and other sleep variables were objectively measured by triaxial accelerometry. Subjects wore an ActiGraph GT3X+ accelerometer (Actisleep, Pensacola, FL, USA) on the non-dominant wrist for 7 consecutive days, 24 h/day (thus including sleeping and waking hours)[37]. Subjects were allowed to remove it only during bathing or swimming, etc. Raw accelerations were recorded using an epoch length of 5 s at a frequency of 100 Hz[38]. During the measurement period, the subjects were required to make daily notes of their in-bed time (time between going to bed and waking) in a diary. Accelerometer assessments were usually completed within the 7 days previous to the PET/CT assessment (see below). The raw acceleration data were exported to csv files using ActiLife v.6.13.3 software (ActiGraph, Pensacola, FL, US), and processed using the GGIR package (v.1.6-0, https://cran.r-project.org/web/packages/GGIR/index.html)[39] in R (v.3.1.2, https://www.cran.r-project.org/). Previously published methods were used to minimize the sensor calibration error (autocalibration of the data based on local gravity)[40], and accelerations were determined by calculating the Euclidean Norm Minus One (ENMO) value as $\sqrt{x^2 + y^2 + z^2} - 1G$ (where 1G ~ 9.8 m/s^2) with negative values rounded to zero. The following were then detected and imputed: i) all non-wear periods, based on the raw acceleration of the three axes, and ii) all sustained, abnormally high accelerations - which are related to the malfunctioning of the accelerometers[39] (see reference[41] for further information). A previously proposed algorithm (validated via polysomnography) was used to combine data from the accelerometers and the subjects' diary reports to detect periods of sleep[42,43]. According to this algorithm, sleep

is defined as any period of sustained inactivity in which there is only minimal arm angle change (i.e., <5º) for 5 min during a period recorded as sleep in a subject's diary[42]. Values for the following sleep-related variables were then determined: i) night onset (time at which the subject fell asleep); ii) wake-up time; iii) in-bed time (time between going to bed and waking up); iv) sleep duration (time between falling asleep and waking up); v) sleep efficiency (ratio of sleep duration to in-bed time); vi) number and duration of periods spent awake after sleep onset (WASO). Daytime naps were not taken into account. Only data from participants who wore the accelerometers for ≥16 h/day over at least 4 days (including at least 1 weekend day) were included in analyses[38].

Sleep quality was determined using the Pittsburgh Sleep Quality Index (PSQI) - a self-rated (via questionnaire), validated, and reliable measurement of this variable that differentiates good from poor sleepers[44]. Subjects responded to 20 items covering seven domains that measure sleep disturbance over the previous month: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleep medication, and day-time dysfunction. In the present work, the scoring system was reversed so that higher values indicated better sleep quality (i.e., fewer sleep disturbances). The scores for the seven domains were then summed[44] to obtain an overall PSQI score on an ascending scale from -21 to 0; this eases interpretation and allows comparisons between studies. Good sleepers were deemed to be those with an overall PSQI score of ≥-5, and bad sleepers as those with an overall score of ≤-6[44].

Sedentary time and physical activity levels

The time spent in sedentary behaviour and in light or moderate-vigorous physical activity was determined using a procedure similar to the above, applying age-specific cut-offs for the ENMO value as previously described[45,46].

Personalized cold exposure and ^{18}F-FDG-PET/CT

The personalized cold-exposure protocol followed, and the quantification of the BAT volume and activity, were as previously reported[41,47,48]. Briefly, subjects sat in a cool room (19.5-20ºC) wearing a water-perfused cooling vest (Polar Products Inc., Stow, OH, USA). The water temperature was reduced from 16.6ºC at ~1.4ºC every 10 minutes until they began shivering (visually detected by evaluators or self-reported).

After 48-72 h had elapsed the subjects went to the Hospital Virgen de las Nieves, where they were again placed in a cool room (19.5-20ºC) and wore the same cooling vest but with the water temperature set ~4ºC above their earlier shivering threshold test result for 2 h. After the first hour the subjects received an injection of ^{18}F-FDG (~185 MBq) and the water temperature was increased by 1ºC to avoid visually detectable shivering. One hour later they were subjected to PET/CT using a Siemens Biograph 16 PET/CT scanner (Siemens, Erlangen, Germany). A low dose CT scan (120 kV) was first performed for attenuation correction and anatomic localization. Immediately thereafter, one static acquisition of 2 PET bed positions (6 minutes each) was performed from the atlas vertebra to the mid chest region[48]. All personalised cold exposure treatments and ^{18}F-FDG-PET/CT data acquisitions were performed according to current methodological recommendations[49].

The BAT volume and activity, estimated via the ^{18}F-FDG uptake, were then determined using the Beth Israel plug-in for the FIJI program[48]. This required: 1) outlining regions of interest (ROIs) in the supraclavicular, laterocervical, paravertebral and mediastinal regions from the atlas vertebra to the 4th thoracic vertebra, using a 3D-axial technique; 2) the determination of the number of pixels in the above ROIs with a radiodensity range of -190 to -10 Hounsfield Units; and 3) the calculation of individualized, standardized threshold ^{18}F-FDG uptake values (SUV) [1.2/(lean body mass/body mass)][49]. BAT volume was determined as the number of pixels in the above range with an SUV value above the SUV threshold. BAT activity was represented as the mean SUV (SUV_{mean}: the mean quantity of ^{18}FDG in the above same pixels) and peak SUV (SUV_{peak}; the mean of the three highest ^{18}F-FDG contents in three pixels within a volume of <1 cm^3). The mean BAT radiodensity was calculated as the mean HU value for the above mentioned ROIs. The SUV_{peak} for the descending aorta (reference tissue) at the height of the 4th thoracic vertebra was also determined, using a single ROI from one slice (image). For confirmatory analyses, the BAT SUV_{mean} and SUV_{peak} with respect to lean body mass (SUV_{LBM})[50] were calculated.

Anthropometry and body composition

Subject height and weight were measured using a SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Lean mass, fat mass, and visceral

adipose tissue (VAT) mass were measured using a Discovery Wi dual energy x-ray absorptiometer (Hologic, Bedford, Massachusetts, USA)[51]. The fat mass index (FMI) was determined as the fat mass (kg)/height squared (m^2), and the lean mass index (LMI) as the lean mass (kg)/height squared (m^2).

Statistical analysis

Descriptive statistics for continuous and categorical variables were used to analyse the subjects' sociodemographic and clinical characteristics. Pearson correlations were performed to examine the association between the studied sleep variables and BAT volume, activity and radiodensity. Partial correlations were then performed to examine the previous relationship after adjusting for sex, and for sex and PET/CT date. One-way analyses of variance (ANOVA), as well as one-way analyses of covariance (ANCOVA) adjusting for sex and PET/CT date, were also performed to examine whether there was any difference in the measured BAT variables based on the number of hours that subjects spent sleeping and on whether they were good or poor sleepers. Pairwise comparisons were performed using Bonferroni post-hoc tests when applicable. Pearson and partial correlation tests were also performed to examine whether sleep variables were related to the ^{18}F-FDG uptake in the descending aorta (reference tissue). As complementary analyses, Pearson and partial correlations were also used to examine whether sleep variables were associated with body composition, before and after adjusting for sex. The level of significance was set at $P \leq 0.05$. All statistical analyses were performed using the Statistical Package for the Social Sciences v.24 (SPSS, Inc. Chicago, IL, USA).

RESULTS

From the initial sample size (participants with complete sleep, ^{18}F-FDG, and body composition data, n=137), 19 participants were excluded due to problems with data collection or analysis (see **Figure S1**). Hence, a final sample of 118 participants (69% women) was included in the main analyses. **Table 1** shows their descriptive characteristics. The participants wore the accelerometers for 6.8±0.5 days, including almost all the night (~99.4% of in-bed time). They slept 6.34±0.73 h per day; and ~52% were classified as good sleepers (score ≥-5). Since the interaction of sex with the determined sleep variables did not have any effect on BAT volume, activity or radiodensity or body composition (P>0.05), all analyses were performed pooling the data for women and men together.

Table 1. Subject characteristics.

	All (n= 118)*		Women (n = 81)		Men (n = 37)	
Age (years)	22	(2)	22	(2)	22	(2)
Professional status, n (%)						
Student	57	(49)	39	(48)	18	(50)
Unemployed	40	(34)	31	(38)	9	(25)
Other professional activities	20	(17)	11	(14)	9	(25)
Body composition						
BMI (kg/m^2)	24.9	(4.7)	23.7	(3.8)	27.5	(5.4)
LMI (kg/m^2)	14.5	(2.4)	13.3	(1.4)	17.2	(2.0)
FMI (kg/m^2)	9.0	(3.0)	9.1	(2.7)	8.7	(3.6)
Fat mass (%)	36.2	(7.3)	38.4	(5.9)	31.3	(7.6)
VAT mass (g)	333.8	(177.7)	284.2	(157.6)	442.3	(172.7)
Objective sleep measures						
Valid days (days)	6.8	(0.5)	6.8	(0.5)	6.7	(0.5)
Non-wear time at night (min/day)	3	(6)	3	(7)	2	(5)
Night onset (hh:mm)	01:16	(01:11)	01:12	(01:11)	01:24	(01:12)
Wake up time (hh:mm)	08:52	(01:03)	08:47	(00:59)	09:03	(01:10)
In-bed time (min/day)	440	(47)	441	(43)	437	(55)
Sleep duration (min/day)	381	(44)	386	(43)	369	(45)
Sleep efficiency	0.87	(0.05)	0.88	(0.05)	0.85	(0.05)
Time in WASO (min/day)	59	(27)	55	(22)	69	(34)
Blocks in WASO (nº/day)	56	(35)	52	(25)	63	(51)
Subjective sleep measures (PSQI)						
Sleep quality	-1.1	(0.7)	-1.1	(0.6)	-1.3	(0.7)
Sleep latency	-1.1	(0.8)	-1.1	(0.8)	-1.2	(0.8)
Sleep duration	-0.8	(0.8)	-0.8	(0.8)	-0.9	(0.8)
Sleep efficiency	-0.5	(0.8)	-0.5	(0.8)	-0.6	(0.8)
Sleep disturbances	-1.1	(0.4)	-1.1	(0.4)	-1.0	(0.3)
Sleep medication	-0.1	(0.5)	-0.1	(0.5)	-0.2	(0.6)
Daytime dysfunction	-0.9	(0.7)	-0.9	(0.7)	-0.9	(0.7)
Global PSQI score	-5.8	(2.6)	-5.6	(2.6)	-6.1	(2.7)
Sedentary behaviour and PA						
Sedentary time (min/day)	794	(65)	786	(55)	812	(80)
Light PA (min/day)	118	(27)	123	(25)	107	(30)
Moderate-vigorous PA (min/day)	89	(32)	92	(31)	84	(34)
PET/CT parameters						
SUV threshold	2.06	(0.23)	2.13	(0.21)	1.90	(0.21)
BAT volume (mL)	68.11	(57.89)	63.72	(52.79)	77.70	(67.53)
BAT SUV_{mean}	3.74	(1.97)	3.96	(2.15)	3.26	(1.40)
BAT SUV_{peak}	11.19	(8.32)	11.71	(8.61)	10.07	(7.66)
BAT radiodensity (HU)	-59.03	(11.76)	-60.21	(11.55)	-56.40	(11.95)
Descending aorta SUV_{peak}	0.80	(0.20)	0.81	(0.21)	0.77	(0.17)

Continuous variables are presented as mean (standard deviation) and categorical variables as number (percentage). *Some data were missing for professional status (remaining cases, n=117), and BAT radiodensity (remaining cases, n=116). BAT: brown adipose tissue, BMI: body mass index, FMI: fat mass index, HU: Hounsfield units, LMI: lean mass index, PA: physical activity, PET/CT: positron emission tomography combined with computed tomography, PSQI: Pittsburgh Sleep Quality Index, SUV: standardized uptake value, VAT: visceral adipose tissue, WASO: awake after sleep onset.

Neither sleep duration nor sleep quality were associated with BAT volume, activity or radiodensity

Figure 1 shows that no objective nor subjective sleep variable was associated with BAT volume, activity (SUV_{mean}, SUV_{peak}) or BAT radiodensity (all P>0.05). Similarly, partial correlations, after adjusting for sex, and for sex and PET/CT date, revealed no sleep variable to be associated with any measured BAT variable (all P>0.05; data not shown). These results remained similar when the analyses were repeated including only those participants with detectable BAT (data not shown). Neither did the results change following additional adjustment for in-bed time, sleep efficiency, non-wear time of the accelerometer during the night, sedentary time, physical activity levels, or any of the body composition variables examined (all P>0.05; data not shown). No changes were appreciated when SUV was normalized to lean body mass (SUV_{LBM}), instead of total body mass (SUV_{BM}) for calculating BAT SUV_{mean} and SUV_{peak} (data not shown).

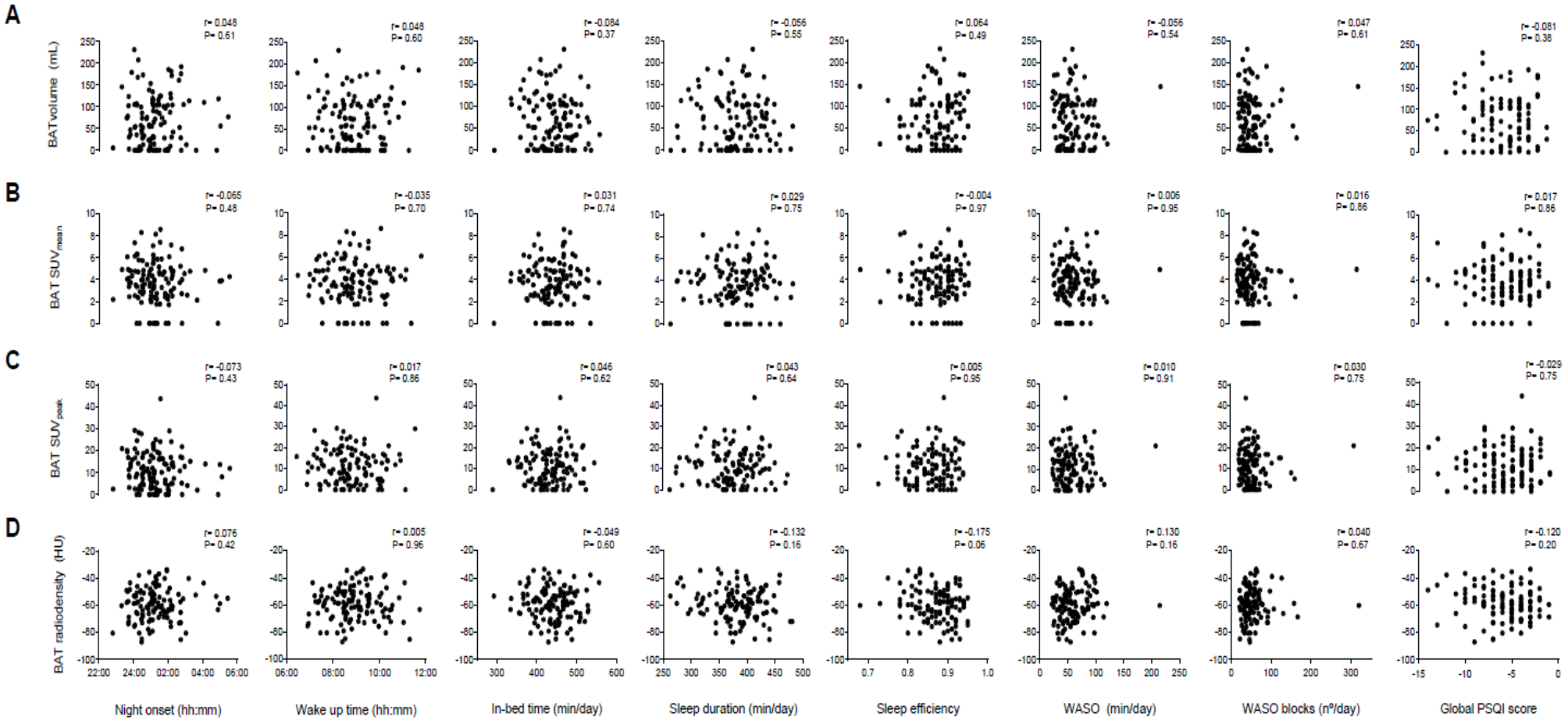

Figure 1. Association between sleep variables and brown adipose tissue (BAT) ^{18}F-FDG uptake (n=118) and radiodensity (n=116). Pearson correlations were performed to examine the association between sleep variables and BAT volume (Panel A), mean standardized uptake value (SUV_{mean}) (Panel B), SUV_{peak} (Panel C), and radiodensity (Panel D). No significant associations were found (P>0.05). Higher global Pittsburgh Sleep Quality Index (PSQI) scores are indicative of better sleep quality.

No differences were found in BAT volume, activity or radiodensity among subjects who slept 4-5 h (n=7), 5-6 h (n=23), 6-7 h (n=67), 7-8 h (n=21), nor between good sleepers (n=61) and poor sleepers (n=57) **(Figure 2)** (all P>0.05). Neither did any appear following adjustment for sex, or for sex and PET/CT date (all P>0.05). It is noteworthy that the subjects in these previous categories had similar body composition and cardiometabolic profile, and undertook similar levels of physical activity (all P>0.05; data not shown). However, the participants who slept for 4-5 h spent considerably longer in sedentary behaviour than those who slept 6-7 h (879 vs. 785 min/day, P<0.001) and 7-8 h (879 vs. 746 min/day; P<0.001). These results remained after grouping subjects as sleeping for 4-5 h or 5-6 h (data not shown).

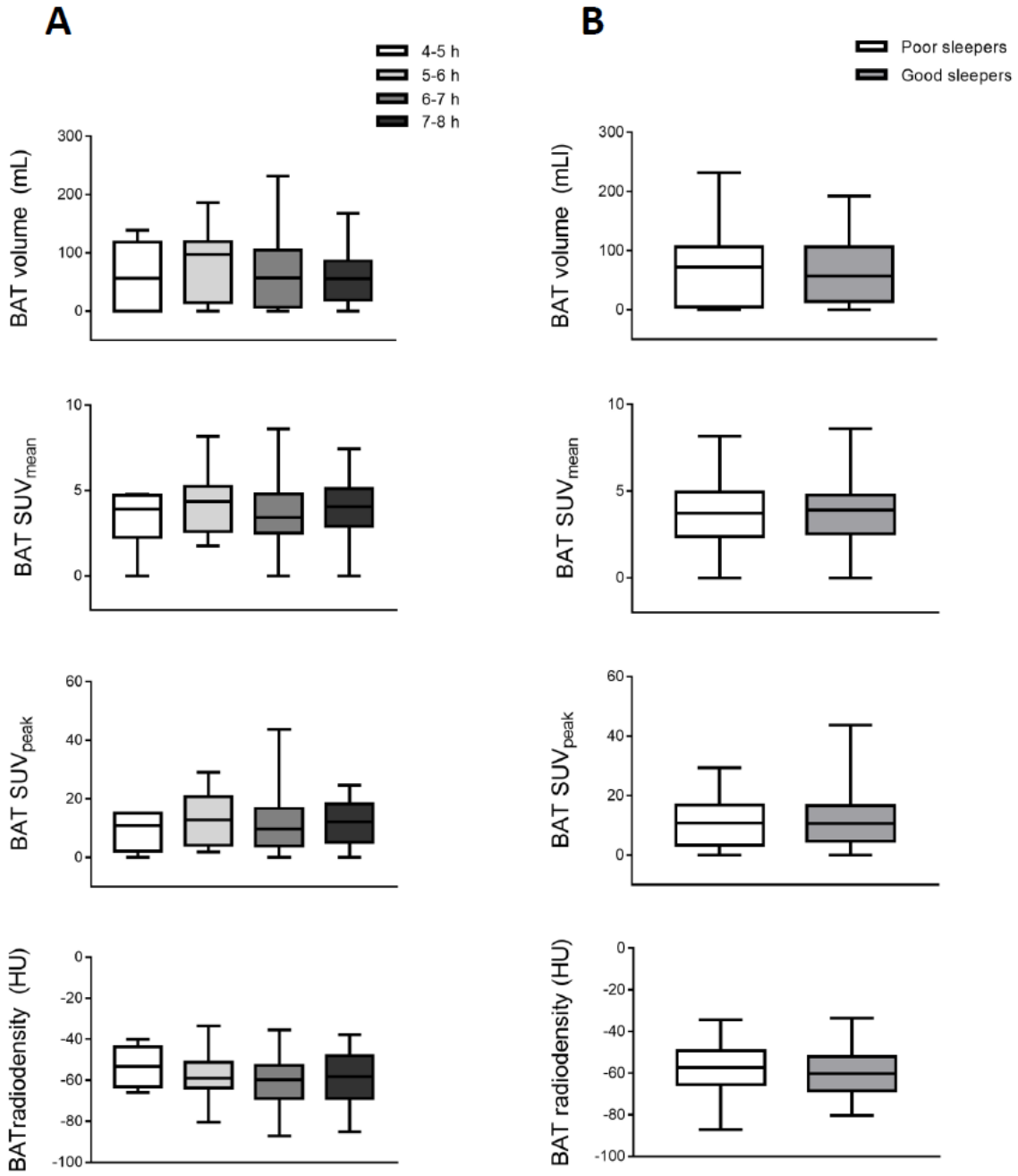

Figure 2. Differences in brown adipose tissue (BAT) volume and activity (determined via ^{18}F-FDG uptake) (n=118) and radiodensity (n=116), based on the number of hours spent sleeping and on whether subjects were good or poor sleepers. **Panel A**: BAT volume, mean standardized uptake value (SUV_{mean}), SUV_{peak}, and radiodensity were compared by one-way analysis of variance (ANOVA) based on the average number of hours per night subjects spent sleeping (measured via accelerometry). Subjects were divided into four categories: those who had 4-5 h sleep (n=7), 5-6 h sleep (n=23), 6-7 h sleep (n=67), 7-8 h sleep (n=21). **Panel B:** BAT volume, SUV_{mean}, SUV_{peak}, and radiodensity were compared by ANOVA based on whether subjects were good or bad sleepers. Good sleepers (n=61) were defined as those who had an overall Pittsburgh Sleep Quality Index (PSQI) score of ≥-5, and bad sleepers (n=57) as those with a score of ≤-6. Measurements of BAT radiodensity were missing for two subjects (one in the 5-6 h sleep time group and one in the 6-7 h sleep time group; and one good sleeper and one poor sleeper). HU: Hounsfield units.

No association was found between any sleep variable and the descending aorta SUV_{peak} value, even after adjustment for sex, and for sex and PET/CT date (all P>0.05**; Figure 3).**

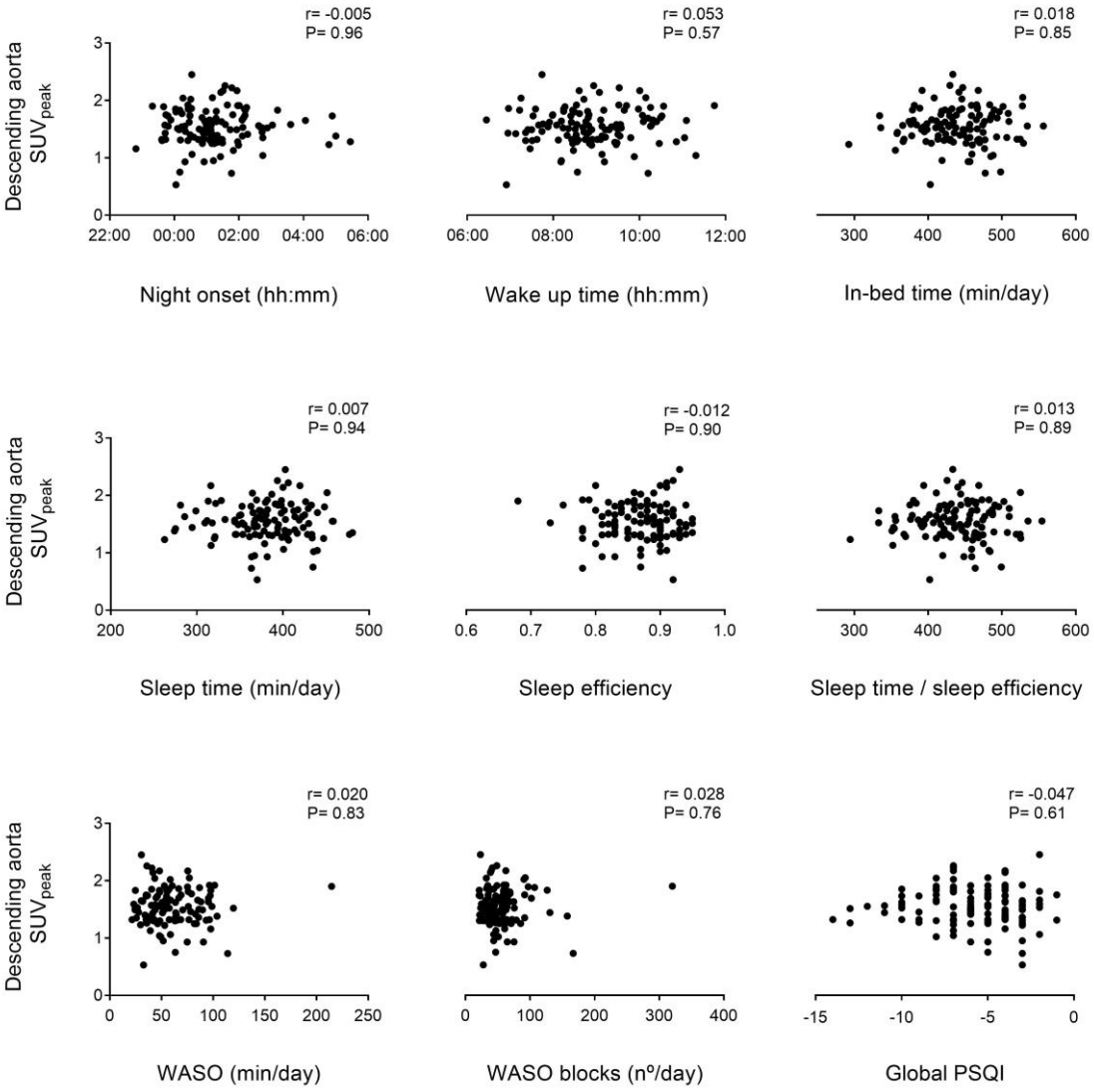

Figure 3. Association between sleep variables and the descending aorta peak standardized uptake value (SUV_{peak}) (n=118). Person´s correlations were performed. Higher values in the global Pittsburgh Sleep Quality Index (PSQI) score are indicative of better sleep quality.

Association between sleep duration and quality and body composition

In-bed time was inversely associated with BMI and VAT mass (r=-0.188, P=0.04 and r=-0.18, P=0.05; respectively), and sleep duration was inversely associated with LMI and VAT mass (r=-0.226, P=0.014 and r=-0.190, P=0.04; **Table 2).** In addition, sleep efficiency and time in WASO were significantly associated with fat mass (r=0.229, P=0.01 and r=-0.269, P=0.003). After adjustment for sex, only in-bed time remained significantly associated with BMI and VAT mass, along with time in WASO with body fat mass (r=-0.189, P=0.041; r=-0.185, P=0.046; and r=-0.186, P=0.045; **Table 2).**

Table 2. Association between sleep variables and body composition (n=118).

	Night onset (hh:mm)	Wake up time (hh:mm)	In-bed time (min/day)	Sleep duration (min/day)	Sleep efficiency	Time in WASO (min/day)	Blocks in WASO (nº/day)	Global PSQI score
BMI (kg/m^2)	0.068	0.086	**-0.188**[a]	-0.173	0.003	-0.049	0.070	-0.054
LMI (kg/m^2)	0.070	0.095	-0.133	**-0.226**	-0.156	0.137	**0.192**	-0.120
FMI (kg/m^2)	0.043	0.055	-0.165	-0.067	0.129	-0.180	-0.045	0.029
Fat mass (%)	0.024	0.012	-0.129	0.026	**0.229**	**-0.269**[a]	-0.151	0.080
VAT mass (g)	0.093	0.067	**-0.183**[a]	**-0.190**	-0.050	-0.011	0.060	-0.117

Pearson´s correlation coefficients are shown. Statistically significant values are shown in bold (P≤0.05). a Indicates associations that remained significant (P≤0.05) after adjusting for sex. BMI: body mass index, FMI: fat mass index, LMI: lean mass index, PSQI: Pittsburgh sleep quality index, VAT: visceral adipose tissue, WASO: awake after sleep onset.

Table S1 shows the relationships among sleep variables measured objectively (by accelerometry) and subjectively (PSQI); the results agree with those of other studies[52].

DISCUSSION

The present results show that sleep duration and quality are not associated with BAT volume or activity (both estimated via ^{18}F-FDG uptake) or BAT radiodensity following cold exposure in young, sedentary adults. These findings persisted after adjusting for sex, PET/CT date and body composition.

Although experiments with rodents indicate sleep homeostatic mechanisms to be closely related to BAT function, evidence for the same in adult humans is scarce. In the single study that exists, Enevoldsen et al.[53] examined whether BAT function was similar in seven patients with narcolepsy type I compared to seven matched healthy controls. Narcolepsy type I is a neurological disorder characterised by the loss of orexinergic neurons; this leads to excessive daytime sleepiness, dysregulated REM sleep, cataplexy, fragmented light sleep, and a higher frequency of sleeping-awake transitions[53,54]. Thus, it is plausible that patients with narcolepsy, who have largely altered sleep patterns, might also show altered BAT function. However, the latter study found that BAT ^{18}F-FDG uptake and sympathetic outflow upon cold exposure were similar in narcoleptic and control participants, calling into question whether sleep duration and quality have any influence over human BAT recruitment and activation. Given the limited sample size, the latter results cannot be generalized to the healthy population, especially since patients with narcolepsy normally have autonomic dysfunction, including changes in their cardiovascular, sympathetic and temperature regulation[55].

No previous studies have examined the relationship between sleep and BAT in healthy adults, precluding any comparison with other results. The present results do not concur, however, with observations made in rodent models revealing an intimate relationship between sleep regulation and BAT thermogenic activity. This disagreement might be explained in several ways. First, there are vast differences between species in terms of their morphology and physiology[56]. Rodents have a smaller body volume to surface area ratio and their thermoregulation system is designed to conserve heat, whereas humans have a larger body volume to surface area ratio and thus dissipate more heat. Hence, rodents have a higher reliance on BAT thermogenic activity during cold exposure than humans[57]. Since the systems that regulate energy balance and metabolic homeostasis are often linked to the neural

circuit that regulates sleep duration and quality[14–18], it would seem coherent that in rodents, in which BAT thermogenic activity makes important contributions to energy balance and metabolic homeostasis, brown adipocyte function should be intimately related to sleep regulation. The same may not hold in humans, however, since recent evidence indicates that the relative contribution of BAT to energy expenditure is rather low, and might be insufficient to impact energy balance[12,13,58]. Secondly, in order to be translatable, experiments in rodents must be performed under conditions that can accurately reflect the physiology and pathophysiology of the humans (e.g., housing mice within their thermoneutral temperature range)[56]. Third, previous studies in rodent models that examined the relationship between sleep regulation and BAT function were performed under different conditions (e.g., following sleep deprivation, the pharmacological or agonist activation of BAT, or in a scenario of systemic inflammation) to those of the present work.

Evidence collected in rodents has shown that their thermoregulatory and sleep mechanisms are closely related[19–21]. Wild-type mice exposed to warm temperatures (35ºC) show a robust increase in NREM sleep[28]. In addition, sleep-promoting mechanisms and BAT thermogenesis are both stimulated by sleep loss, being positively correlated[22,28]. Similarly, in adult humans, an increase in the distal skin temperature during the night (which is phased-opposed to the decrease in core temperature)[23], is associated with shortened sleep latency and increases in sleep duration and depth[24,25], demonstrating a link between the thermoregulatory and sleep centres. Accordingly, we previously determined in a sub-cohort of the present subjects (n=77) that the time at which this increase happens is weakly related to BAT activity (reflected as SUV_{mean}) (B=-0.2, P=0.04; paper submitted). These findings, together with the present results, suggest that sleep duration and quality might not be directly related to BAT activity following cold exposure. In addition, rodent experiments have shown that BAT may act as a sleep-promoting signalling organ; there are extensive afferent projections running from the BAT into the hypothalamic area, and a population of these is thermosensitive, raising the possibility that BAT thermogenesis may induce sleep independent of changes in core temperature[14,22,28,29]. This hypothesis agrees with the fact that there seems to be a significant time lag between the somnogenic and delayed body temperature effects following pharmacological activation of BAT in rodents[28]. Whether

BAT might act as a sleep-prompting signalling organ in humans remains to be seen. Nor can it be ruled out that human BAT might exert its function on sleep regulation via endocrine mechanisms. For instance, BAT secretes adenosine[59], an endogenous factor that promotes sleep by blocking inhibitory inputs to the ventrolateral preoptic area's sleep-active neurons[60], and can express factors such as interleukin-6, interleukin 1, and tumour necrosis factor-alpha, all of which have somnogenic effects[7,28].

A complementary aim of the present work was to examine whether sleep duration and quality are associated with obesity and body composition. The results show a weak inverse relationship between in-bed time and both BMI and VAT mass (after controlling for sex). Interestingly, in-bed time and sleep duration were moderately and inversely associated, whereas the moment of entering sleep was positively associated with the time spent in sedentary behaviour (**Table S2**). This suggests that those subjects who slept less, and who went to sleep later, where those who spent more time in sedentary behaviour. This may be explained in that sleep curtailment, or a late chronotype (which is related to misalignment between social rhythms and the circadian clock), may be related to greater drowsiness during the day, and consequently to more sedentary behaviour and an increased risk of obesity[61]. Therefore, the in-bed time may be related to increased risk of obesity through indirect mechanisms. Taking everything into account it is tempting to speculate that the relationship between sleep curtailment and risk of obesity might not be influenced by BAT volume or activity. Other behavioural (e.g., sedentary time) and physiological mechanisms related to homeostatic (e.g., sleep pressure), circadian (e.g., sleeping-awake cycle schedule) and metabolic control (e.g., dysregulated secretion of gastrointestinal peptides, alterations to the appetite regulation centres of the brain) may explain this relationship better.

The present results should be interpreted with caution; the study has a cross-sectional design that precludes the establishment of causal relationships. For instance, it might be possible that habitual short sleep and poor sleep quality could alter the function of the BAT metabolism, as it has been previously shown with other metabolic functions (e.g., glucose metabolism)[2]. In contrast, it could be hypothesized that BAT exerts its influence on sleep duration and quality. Anyhow, sleep is a complex phenomenon, which is influenced by behavioural, but also by physiological

mechanisms related to homeostatic, circadian and metabolic control under the participant´s natural sleep environment. Therefore, it exits the possibility that these factors could be influencing the relationship between sleep parameters and ^{18}F-FDG uptake and radiodensity. Further, the sample was composed of young adults, most of whom had a healthy cardiometabolic profile (data not shown); this could have masked or weakened the associations between sleep variables and BAT ^{18}F-FDG uptake, BAT radiodensity or obesity risk. It should also be remembered that the use of the shivering threshold (subjectively assessed) as the end-point of the personalized cooling protocol may have introduced variation into the cooling stimulation, which would be reflected in the subjects' BAT activation[62]. Despite being the most used technique to assess BAT, a single static ^{18}F-FDG PET-CT scan has several limitations that might not allow for the accurate estimation of cold-induced BAT metabolic activity[63]. Whether the present findings will be replicated when using other radiotracers such as ^{15}O-oxygen, ^{11}C-acetate or ^{18}F-fluoro-6-thia-heptadecanoic acid (^{18}FTHA) to quantify BAT metabolism, remains to be seen. It is also necessary to consider that: i) napping time was not included in the analyses, since we do not have information that allows an accurate quantification of it. The timing and duration of napping could have a profound effect on night sleep, and might partially mask the relationship between sleep parameters and BAT ^{18}F-FDG uptake and radiodensity. However, based on the acceleration records and participant reports, it seems that most of our participants did not nap during the day (also probably because many of them were university students and had to attend classes); ii) although accelerometer records (combined with sleep diaries and subjective measures) are a valid and extensively used measure of sleep duration and quality under free-living conditions[43,64], they are not able to differentiate between REM and NREM sleep, and thus they may provide a limited insight into the real architecture of sleep wake-activity; iii) although we followed the most updated international recommendations[49] to quantify and analyse BAT ^{18}F-FDG uptake, we performed an unique temporal measure after a personalized cold exposure. Therefore, future studies should examine how continuously measured BAT activity is specifically related to REM and NREM sleep using polysomnography records (since these phases are metabolically different[34]), in order to get a deeper insight into the interaction between BAT function and sleep regulation. This fact will be conditioned by the

advance of the current technologies to assess BAT metabolic activity in a non-invasive and non-ionizing manner, or by the validation of indirect markers that accurately reflect its activity. Furthermore, experimental studies should manipulate sleep (e.g. sleep deprivation) and/or BAT function (e.g., use of beta-3 adrenergic agonists available) under well-controlled lab conditions in order to stablish a causal relationship.

In conclusion, sleep duration and quality appear not to be related to BAT volume or activity (both estimated by ^{18}F-FDG uptake) or BAT radiodensity following cold exposure, in young healthy, sedentary adults. Further studies are needed to fully understand the underlying mechanisms of sleep regulation, and how short sleep duration and poor sleep quality are related to the obesity pandemic and the increase in cardiometabolic disease.

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SUPPLEMENTARY MATERIAL

Table S1. Association between objectively measured (accelerometry) and subjectively measured (Pittsburgh Sleep Quality Index [PSQI]) sleep variables (n = 118).

		Night onset (hh:mm)	Wake up time (hh:mm)	In-bed time (min/day)	Sleep duration (min/day)	Sleep efficiency	Time in WASO (min/day)	Blocks in WASO (nº/day)
PSQI variables	Sleep quality	0.053	-0.055	-0.108	-0.071	0.067	-0.097	-0.136
	Sleep latency	-0.008	-0.030	-0.045	-0.118	-0.084	0.054	0.053
	Sleep duration	0.011	**0.198***	**0.298*****	**0.208***	-0.158	**0.226***	**0.227***
	Sleep efficiency	-0.072	**-0.219***	-0.125	-0.156	-0.062	0.041	-0.032
	Sleep disturbances	0.116	0.008	-0.035	-0.030	-0.059	0.072	0.015
	Sleep medication	-0.024	-0.057	0.035	-0.079	-0.144	0.145	0.138
	Daytime dysfunction	0.011	0.084	0.136	0.106	-0.056	0.062	0.084
	Global PSQI score	-0.007	-0.010	0.085	0.019	-0.101	0.113	0.077

Spearman correlation coefficients are shown. Statistically significant values are shown in bold (*P≤0.05, ***P≤0.001). WASO: awake after sleep onset.

Table S2. Association between sleep variables and the time spent in sedentary behaviour, and in different physical activity (PA) intensities (n=118), before and after adjustment for sex (Model 1 and Model 2 respectively).

		Night onset (hh:mm)	Wake up time (hh:mm)	In-bed time (min/day)	Sleep duration (min/day)	Sleep efficiency	Time in WASO (min/day)	Blocks in WASO (nº/day)	Global PSQI score
Model 1	Sedentary time (min/day)	**0.355*****	-0.027	**-0.589*****	**-0.530*****	0.054	-0.169	**-0.227***	0.028
	LPA (min/day)	0.087	-0.137	**-0.225***	-0.116	0.121	**-0.208***	-0.043	-0.049
	MVPA (min/day)	0.046	-0.072	-0.095	-0.060	0.048	-0.069	0.066	-0.093
Model 2	Sedentary time (min/day)	**0.347*****	-0.051	**-0.593*****	**-0.513*****	0.105	**-0.224***	**-0.260****	0.045
	LPA (min/day)	0.114	-0.111	**-0.244****	-0.174	0.060	-0.154	-0.006	-0.074
	MVPA (min/day)	0.055	-0.060	-0.099	-0.081	0.023	-0.045	0.082	-0.103

Pearson and partial correlations coefficients are provided for Models 1 and 2, respectively. Statistically significant values are shown in bold (*P≤0.05, **P≤0.01, ***P≤0.001). LPA: light physical activity, MVPA: moderate-vigorous physical activity, PSQI: Pittsburgh Sleep Quality Index, WASO: awake after sleep onset.

Figure S1

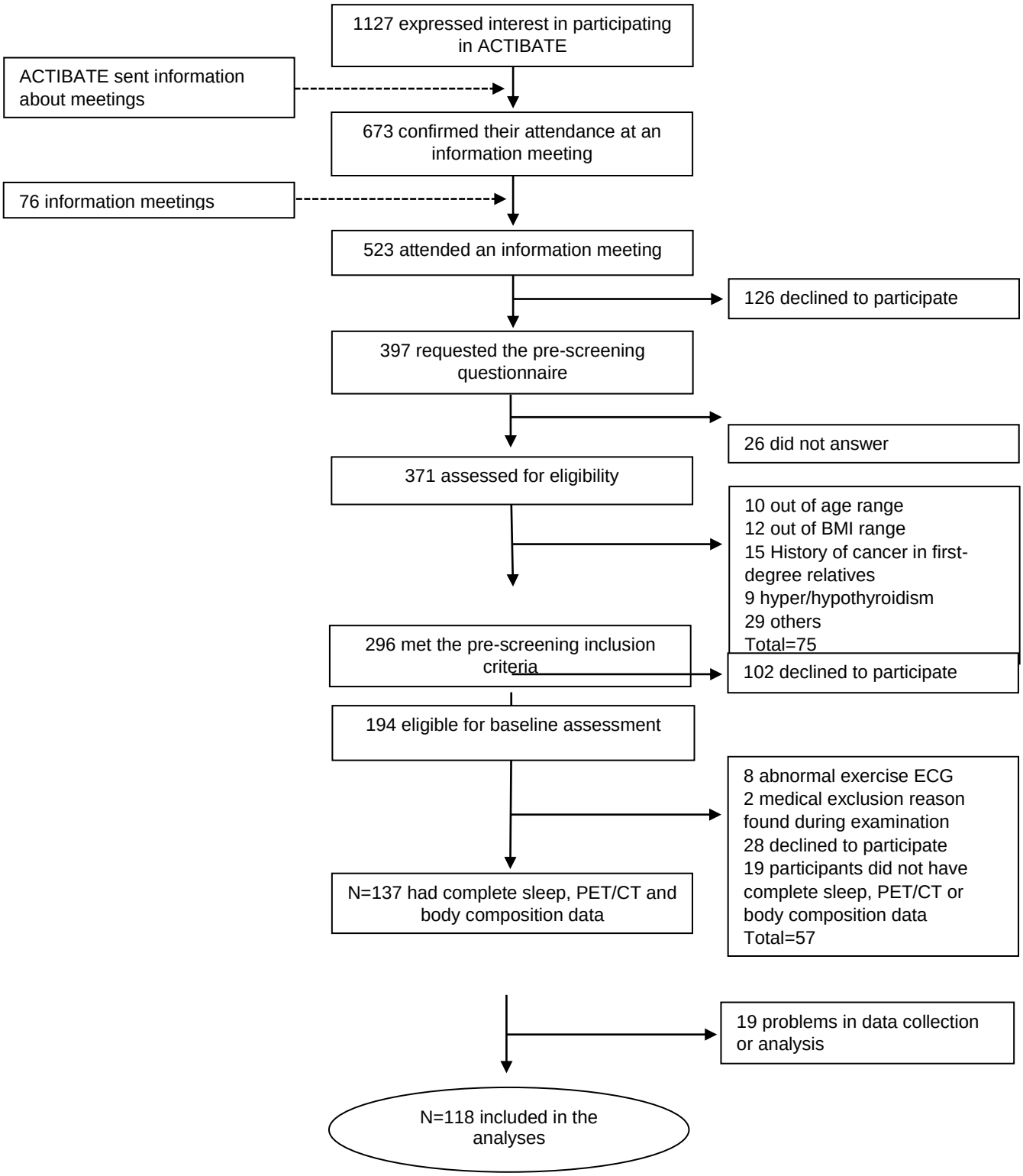

Figure S1. Flow-chart for subject enrolment. BMI: body mass index, ECG: electrocardiogram, PET/CT: positron emission tomography combined with computed tomography.

PART III. Exercise and physical activity as new strategies to recruit and activate brown adipose tissue

STUDY V

Effect of an acute bout of aerobic exercise on UCP1 and IL-6 protein concentrations in brown adipose tissue of wild-type mice

ABSTRACT

Exercise has been proposed as a potential stimulus in the recruitment and activation of brown adipose tissue (BAT), through the sympathetic activity increase, and the release of adrenergic independent factors. Nevertheless, how human BAT is regulated during exercise is poorly understood. Indeed, no studies have examined which is the effect of acute exercise on human BAT.

As a first step before translating to humans, in this study we aimed to examine the acute effect of aerobic exercise on the expression of several genes related to thermogenesis and metabolism in the interscapular BAT (iBAT) in wild-type mice. Adult (8-week old) male C57BL/6J mice, fed with a standard laboratory ad libitum (chow diet), were randomly assigned to the non-exercise or exercise group. Mice within the exercise group were familiarized during 3 different days with a running treadmill, and then, they underwent an incremental exercise test. During the incremental exercise protocol, the speed was constantly increased, until mice were exhausted or met 90 min. Then, they were sacrificed and dissected, and the organs of interest - i.e, the iBAT and subcutaneous and epididymial white adipose tissue (sWAT and eWAT) - were collected and frozen in liquid nitrogen. Western-blot assays were performed to determine differences between the non-exercise and exercise mice, in the levels of UCP1, PGC1α, p-p38 MAPK, IL-6, IL-6Rα, STAT, and pSTAT3 in iBAT.

Our results showed no differences in UCP1, IL-6, IL6R-α, STAT3 and PSTAT3 between non-exercise and exercise mice in iBAT. However, when total loaded protein was increased, IL-6 levels seemed to increase in a dose-dependent manner, and seemed to be higher in exercise mice than non-exercise mice. Of note, p-P38 MAPK could not be detected in any of the groups. Taken all together, an acute bout of aerobic exercise does not seem to have an effect on UCP1 levels in iBAT through the canonical activation pathway, neither through a new explored one, the IL-6/JAK/STAT3 pathway. However, during acute aerobic exercise, iBAT might be producing small amounts of IL-6, and future studies should examine whether it is being secreted with an endocrine function.

INTRODUCTION

The study of exercise offers enormous potential as a strategy to face or prevent the apparition of a wide range of diseases, including obesity, cardiometabolic diseases, dyslipidemia, depression, and certain tumours[1,2]. However, the underlying mechanisms by which exercise induces many of its benefits are not completely understood[3].

Exercise has been proposed as a potential stimulus in the recruitment and activation of brown adipose tissue (BAT)[4–6]. It has been hypothesized that exercise might be able to activate and recruit human BAT through several mechanisms, such as the canonical sympathetic activity increase, and the release of adrenergic independent factors[4,6]. However, experiments are scarce[7–11], and there is not clear evidence yet whether exercise modulates BAT function or not. To date, evidence collected from chronic exercise interventions in mice is highly controversial[12–23] - probably due to the lack of standardization, different types and characteristics of exercise stimulus, confounding factors (e.g., HFD, low room temperature), and bad scientific methodology[16]. In humans, findings from case control studies[8,10] have shown that trained individuals have lower cold-induced BAT volume and activity than sedentary or untrained individuals, as estimated by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography combined with computed tomography (PET/CT). Similarly, a pilot study[7] reported lower BAT activity in trained versus sedentary control participants. Recently, Motiani et al.[9] also showed that 2 weeks of exercise (cycling) decreased insulin-stimulated BAT ^{18}F-FDG uptake in participants with active BAT prior to the intervention. Taken together[7–10,24,25], it seems that exercise is associated with the downregulation of BAT glucose metabolism, although further studies are needed to better understand the exercise-induced adaptations of human BAT.

Importantly, to examine which are the effects of acute exercise on the physiological and molecular interface of human BAT is of great interest, since it would allow us to further understand how this tissue is regulated, and we could better interpret BAT adaptations to chronic exercise. However, to date, no experiments have examined this issue. This scientific gap is explained because of the lack of non-invasive in vivo techniques that allow to monitor BAT activity in real time during exercise, and its anatomical characteristics – which greatly difficult obtaining tissue samples through

biopsy techniques[26]. Before trying to translate findings from exercise chronic interventions to humans, it is fundamental to determine whether acute exercise can modulate BAT function under well-controlled lab conditions, and if so, through which mechanisms acute exercise is able to modulate it (classical and alternative mechanisms). A potential approach to initially address this issue might be to conduct experiments in rodents, and subsequently to perform studies with specific targets in humans (whenever possible).

Of note, during exercise, the skeletal muscle secretes specific factors to the bloodstream, known as myokines, which exert their functions in different organs and tissues[27]. It is possible that some of these myokines (e.g., IL-6, meteorin-like, musclin, and irisin) have a potential role modulating BAT activation and recruitment[28–30]. Similarly, exercise induces several physiological changes in white adipose tissue, including the release of adipokines (such as leptin or FGF21), that might also modulate BAT activity. In fact, during exercise, the organ cross-talk or communication become even more intense in an attempt to restore energy homeostasis and satisfy all our organisms needs. Therefore, to explore whether these adrenergic independent mechanisms might be modulating BAT activity is crucial.

Of special interest is the IL-6, a central mediator on metabolic and inflammation processes[31]. Its release can prompt an increase of catecholamine secretion, and therefore it may be able to provoke changes in BAT activity through the sympathetic-adrenal axis[32] - or other unexplored via. Previous evidence[33] has shown that IL-6 is expressed in primary cultures of mouse brown adipocytes, and that IL-6 expression increases 40-fold in response to norepinephrine. In addition, when stimulated by β3-adrenergic receptor (β3AR) agonists, brown adipocytes secrete IL-6 to the culture media, suggesting that, under noradrenergic stimulation, BAT may contribute to systemic IL-6 concentrations[33]. Furthermore, IL-6 gene central delivery into the hypothalamus of rats, showed that the chronic overexpression of IL-6 enhanced UCP1 protein levels in iBAT- and this was dependent on sympathetic innervation[34]. Similarly, IL-6 gene transfer – through hydrodynamic delivery – showed that the persistent IL-6 gene expression enhanced gene expression and increased the protein levels of UCP, PGC1α, and pSTAT3, in HFD induced obese mice[35]. Stanford et al.[36] also showed that BAT transplantation from donor mice improved glucose homeostasis and insulin

sensitivity in recipient WT mice, but this effect was lost when the transplanted BAT was obtained from IL-6 knockout mice. All this evidence together calls into attention the role of IL-6 mediating the thermogenic activity of BAT, and its effects on metabolism. As during acute exercise, systemic IL-6 concentrations are generally increased[37,38], its remains to be explored whether this could be a mechanism linking exercise and BAT function. To mention also is that a posterior study by Knudsen et al.[39], showed that IL-6 is required for an exercise training-induced increase in inguinal WAT UCP1 mRNA content, suggesting that IL-6 may also have implications in the browning of WAT.

Therefore, we aimed to examine the effect of an acute bout of aerobic exercise on the protein levels of UCP1 and IL-6 in BAT, in wild-type mice. In addition, we examined whether IL-6 could be implicated in an alternative pathway to activate BAT.

METHODS

Experiments were conducted on adult (8-week old) male C57BL/6J mice, purchased from Monash animal research platform (MARP, Australia), and housed under standard conditions. More specifically, mice were kept in cages (always in pairs) at 23ºC, under controlled lighting conditions in all cases (12/12 light-dark cycle, from 7:00 to 19:00). Mice were fed with a standard laboratory ad libitum - chow diet, (20% protein, 8.5% fat, 25% starch, Ridley Agriproducts, Melbourne, Australia). All procedures performed in mice were approved under ethics application 18981 by the Monash Institute of Pharmaceutical Science (MIPS) AEC in the Faculty of Pharmacy and Pharmaceutical Sciences.

Procedures

Mice were randomly assigned to the non-exercise or exercise group. Mice within the exercise group were familiarized during 3 different days with the running treadmill. The protocol for this familiarization period was a follow: on day 1, mice were put into the treadmill (always in groups 5 mates), and ran 10 m/min for 10 min. Then, the total exercise load was increased, so that on the 2nd and 3rd, they respectively ran 12 m/min for 15 min, and 14m/min for 20 min. Then, mice had at least 3 days for recovery. After the recovery period, the exercise test was performed. An incremental protocol was applied, starting at 10m/min, with no inclination, and increasing the speed 2m/min. Of

note, for those mice who stopped running, or have difficulties to follow the selected speed, a tooth brush was used to motivate them to run. Mice exercised for 90 min or until exhaustion - defined as the inability to continue at the selected speed despite gentle encouragement with a tooth brush for 5-10s.

Given that we were interested on the acute changes of aerobic exercise, when mice were exhausted or met the pre-stablished running time, they were sacrificed and dissected, and the organs of interest - i.e, interscapular BAT (iBAT), and subcutaneous and epididymial white adipose tissue (sWAT and eWAT) - were collected and frozen in liquid nitrogen. Then, they were stored at -80ºC freezer. A similar procedure was followed for those mice who did not perform exercise.

Western Blotting analysis

Tissues were chipped on dry ice, and kept into 2mL tubes. Approximately, 50mg of interscapular BAT, and 100 mg of sWAT and eWAT, were taken from each mouse. Each tissue sample was homogenized in a 0.5 mL lysis buffer [50 mM Tris (pH, 7.4), 130 mM NaCL, 5mM EDTA, NP-40 (1%), mixed with 1M NaF, 100 mM PMSF, and 50ul Protease inhibitor (1:100 sigma#P5726)], using an electrical homogenizer. Then, tissue samples were centrifuged at 13000 rpm for 30 min, and the supernatant was transferred to new tubes, where it was diluted with molecular water (1/40 for iBAT, 1/5 for sWAT and eWAT). Then, a BCA (Pierce method) protein assay was performed to determine the total protein concentrations.

Total protein (except indicated, always 30 ug/well) was resolved with sodium dodecyl sulfatete-polyacrylamide gel electrophoresis (SDS-page) on 10% or 12% acrylamide/bis-acrylamide gels using a Trans-Blot Turbo Transfer System (BioRad, Berkley, California), and transferred to nitrocellulose membranes (BioRad, Berkley, California). Membranes were blocked in a Tris-buffered solution containing 10% non-fat milk for 1 h. Membranes were then incubated (4ºC overnight with shaking) first with primary antibodies specific for UCP1 (#14670), p-p38 MAPK (#9211), IL-6 (#12912), STAT3 (#4904), pSTAT3 (#9131) from Cell Signalling (Boston, MA), PGC1α (#sc-13067) and IL-6Rα (sc-13947) from Santa Cruz Biotechnology (Santa Cruz, CA), and β-tubulin (#ab6046) from Abcam (Cambridge MA).

After washing, the membranes were incubated in a 1:2000 dilution of a secondary anti-rabbit antibody at room temperature for 1 h, in a TBST solution containing 2.5% BSA. Protein bands or signals were detected using a chemiluminescence horseradish peroxidase substrate (ThermoFisher), and a SuperSignal Western Blot Enhancer (ThermoFisher) was added when signal intensity and sensitivity needed to be increased. UCP1 protein bands signals were expressed relative to β-tubulin signal, and the background pixel density was deducted from the band values. Digitized images were quantified with the software on Beth Israel plugin for FIJI http://sourceforge.net/projects/bifijiplugins/[40], using ImageJ (USA).

Statistical analyses

A statistical analysis was performed using the Student's t test for independent samples. Data were reported as individual values, or as mean ± standard deviation. The level of significance was set at $P<0.05$. Statistical trends were reported as $P>0.05$ and ≤ 0.1. The statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS version 24, Inc. Chicago, IL, USA).

RESULTS AND DISCUSSION

As a proof-of concept experiment, we firstly examined whether UCP1 protein was present across adipose tissue depots as previously evidenced. Accordingly, we found that UCP1 protein levels were higher in the iBAT compared to sWAT and eWAT (**Figure 1).** In addition, we found that UCP1 protein concentrations seemed to be higher in the sWAT, an area susceptible of browning, compared to the epididymial WAT, which is composed mainly by pure white adipocytes.

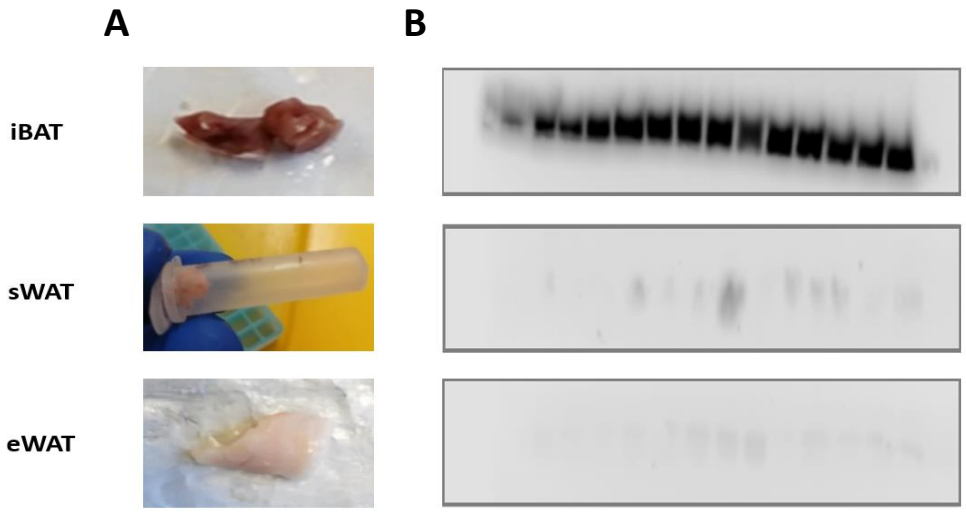

Figure 1. Proof of concept- experiment. Panel **A** shows small pieces of interscapular BAT (iBAT), subcutaneous/inguinal WAT (sWAT), and epididymial WAT (eWAT) which were later cut for biochemical analyses. Panel **B** shows the UCP1 protein concentrations across the previous tissues, in wild-type mice.

Then, we aimed to examine whether an acute stimulus of aerobic exercise could affect the levels of proteins related to thermogenic and metabolic functions across adipose tissue depots. With that purpose, we analysed UCP1 and PGC1α protein concentrations in iBAT, sWAT, and eWAT, in non-exercise vs. exercise mice. We observed that after an acute bout of aerobic exercise, there was a trend for UCP1 levels to be lower in exercise mice compared to non-exercise mice (**Figure 2A)**. However, when we relativized UCP1 to β-tubulin protein concentrations, and statistically compared both groups, there was no significant difference between groups (P=0.1, **Figure 2B).** Of note, 2 outlier mice showed elevated concentrations of UCP1 within the exercise group, which might be explained because the duration of the experimental protocol differed among mice - not of all them were similarly motivated to run.

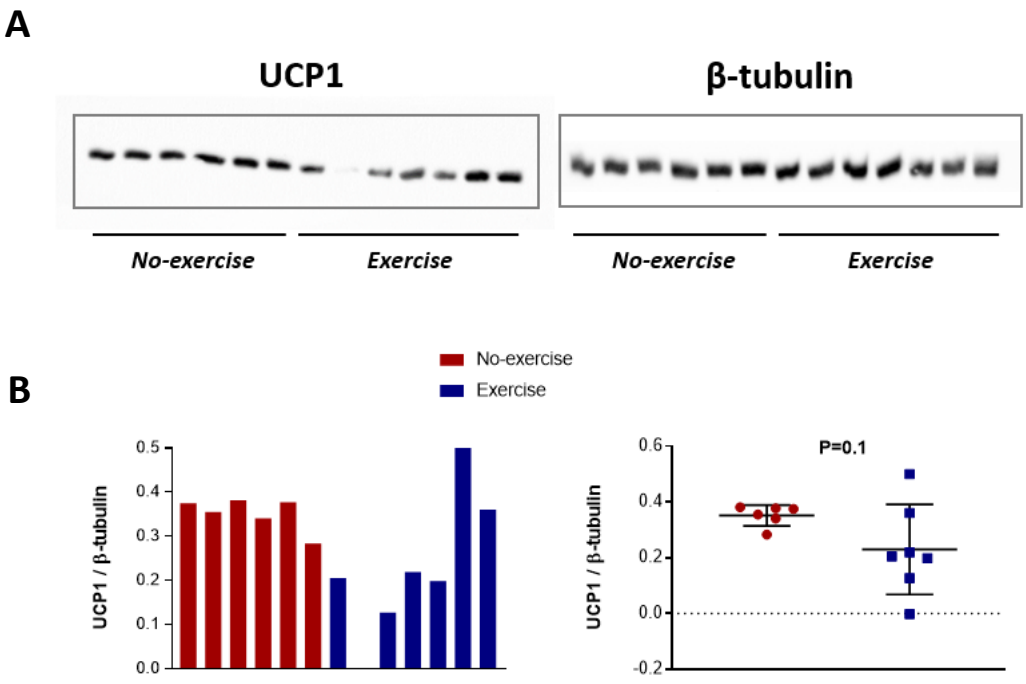

Figure 2. UCP1 protein concentrations in the interscapular BAT (iBAT) of non-exercise (n=6) versus exercise (n=7) wild type mice. Panels **A** shows the blots with the protein concentrations of UCP1 and β-tubulin (reference protein). **Panel B** shows the individual values of UCP1 protein concentrations relativized to β-tubulin, in non-exercise versus exercise mice, and the mean and standard deviation of for each one of the groups. Statistical comparisons were performed using a t-Student test for independent samples.

We additionally analysed the UCP1 protein concentrations in the sWAT and eWAT. Nevertheless, the blots from these analyses were a bit blurred, and some noise could be observed at the background. Hence, they were not included in this section, although it seems that there were no differences between groups (data not shown). Regarding the protein concentrations of PGC1α across adipose tissue depots, the applied antibody did not have a good specificity for this molecule, and consequently these results were omitted since they were not valid.

Furthermore, we investigated whether there was any difference between the no-exercise and exercise mice in the phosphorylated p38-MAPK, an important transductor in the canonical pathway for activation of BAT[41] (**Figure 3)**. However, we were not able to obtain any signal either in the blots, or when we performed a replicate. This seems to confirm that acute aerobic exercise might not influence UCP1 expression through this pathway.

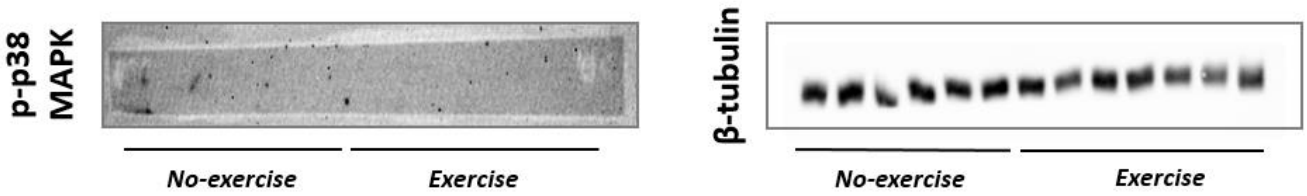

Figure 3. p-p38 MAPK (phosphorylated-p38 mitogen activated protein kinase) and β-tubulin (reference) protein concentrations, in the interscapular BAT of non-exercise (n=6) versus exercise (n=7) wild-mice.

Secondly, we aimed to examine whether the systemic release of IL-6 during an acute bout of aerobic exercise, could have any effect on the UCP1 levels in iBAT through an alternative signalling pathway – the IL-6/JAK/STAT3 pathway **(Figure 4).**

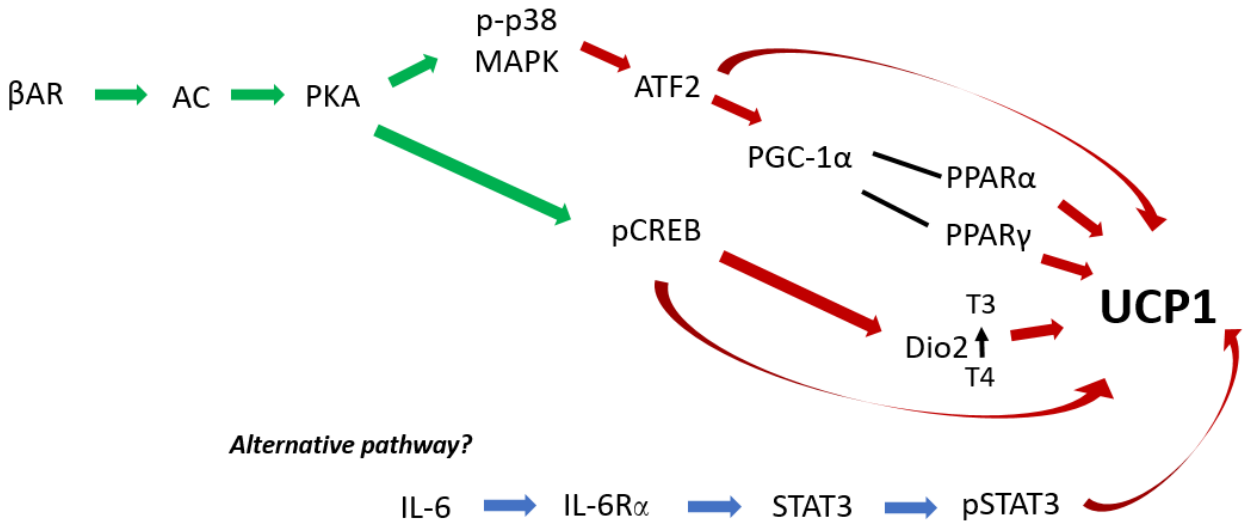

Figure 4. Theoretical model by which the IL-6 produced/secreted during acute aerobic exercise could have an effect on BAT function. We aimed to test the pathways described by the blue arrows. Green arrows refer to transduction of signal through phosphorylation, or to the interaction between proteins, whereas red arrows indicate transcription regulation processes.

When we examined the IL-6 protein concentrations in iBAT (30ug/well of total protein), we did not find any signal for the bands. This could be explained mainly because: i) it was not, or it was minimally present in iBAT; ii) the total protein concentrations that we used in the Western Blot analyses were too low for IL-6 to be detectable; iii) some methodological problem, such as that we chose an antibody with low specificity for this molecule, the blocking was not efficient, etc. Hence, we performed the same analyses, but instead of using a total protein mass of 30ug, we used 60 and 80ug, in independent membranes. Interestingly, we were then able to identify IL-6 in iBAT (in a dose-dependent manner, **see Figure 5B),** and IL-6 levels seemed to be higher in both cases in those mice who performed exercise than in no-exercise mice. In addition, our results showed that IL-6Rα seemed not to be different between both groups **(Figure 5A)**. Taken together, it could be speculated that: i) systemic IL-6 is not entering iBAT (at least through the classical signally pathways, but maybe by the trans-signalling pathway); ii) IL-6 is being produced (at small amounts) in iBAT.

To understand whether this pathway may have some functional implications on the UCP1 levels of iBAT, we further examined whether STAT3 and pSTAT3 protein levels were different in both groups (**Figure 5)**. Regarding STAT3, it could be detected in the blots, although we did not obtain a good-quality band, and therefore no comparisons between non-exercise and exercise mice were performed. It could be also observed the characteristic presence of some dimers in these blots - likely with other members of the STAT family. On the opposite side, we did not obtain any signal for pSTAT3 levels. This seems to indicate that STAT3 protein was present in iBAT, in both non-exercise and exercise mice, but that after exercise, STAT3 was not phosphorylate; therefore, exercise may not have any influence on the regulation of UCP1 expression through this via.

Hence, it seems that acute aerobic exercise is not likely to have any effect on UCP1 protein levels through the IL-6/JAK/STAT3 pathway in wild-type mice. Nevertheless, it is tempting to speculate that during exercise iBAT may produce IL-6 at small amounts. Since this IL-6 is not systemic (no differences in IL-6Rα), and IL-6 does not seem to have any role on the thermogenic function of BAT, further evidence

should examine whether this batokine[42] is secreted - as previously shown in culture media[33] - to the blood torrent and may have some endocrine function (e.g., as an energy sensor, increasing insulin--stimulated glucose uptake in other tissues, etc.).

Some limitations should be considered in the present study. Firstly, this is an exploratory study which will help to lead future research. The number of mice used, as well as of experiments required, will be larger in order to corroborate these findings. Secondly, we focused on the main molecules or mediators of the pathways of our interest. Although this is enough to test whether an acute bout of aerobic exercise may have functional implications on UCP1 regulation, a more comprehensive and detailed characterization of these pathways may be necessary. Mice were caged at 23ºC approximately, imposing a thermal stress (cold) to them[43], which may have affected our results.

In conclusion, an acute bout of aerobic exercise does not seem to have an effect on UCP1 levels in iBAT through the canonical activation pathway, neither through a new explored one, the IL-6/JAK/STAT3 pathway. However, during acute aerobic exercise, iBAT might be producing small amounts of IL-6, and future studies should examine whether it is being secreted with an endocrine function.

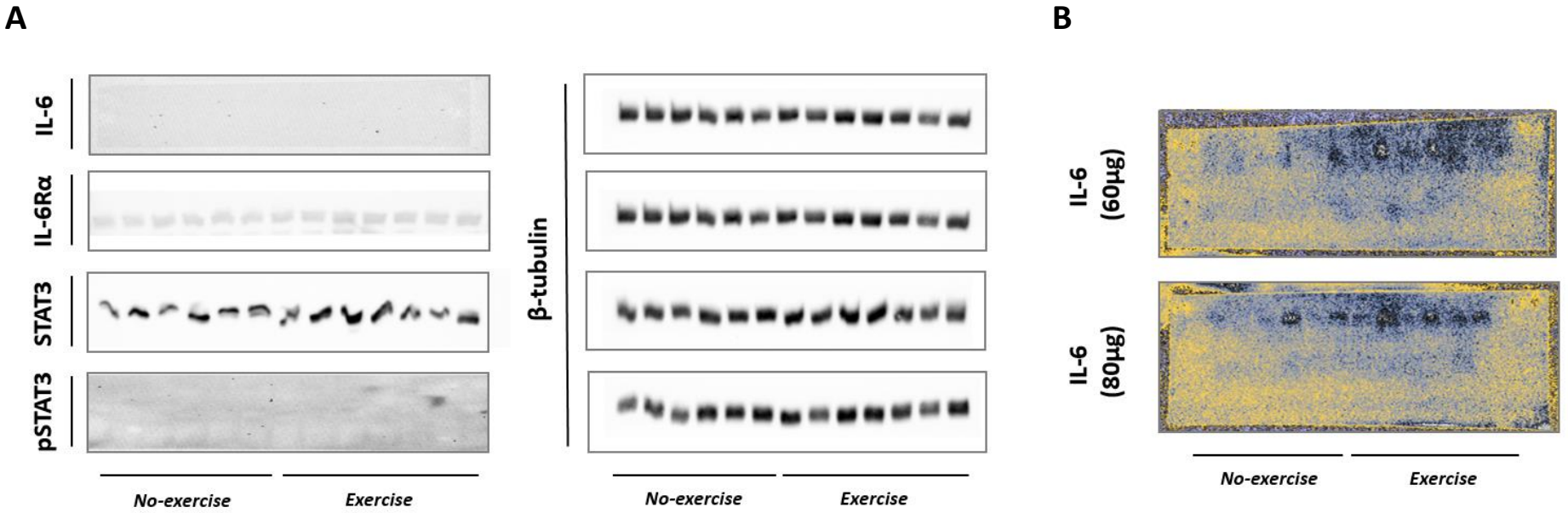

Figure 5. Protein concentrations of IL-6 (interleukin 6), IL-6Rα (interleukin 6 receptor alpha), STAT3 (signal transducer and activator of transcription 3), pSTAT3 (phosphorylated STAT3), and β-tubulin (reference protein), in the interscapular BAT of no-exercise (n=6) versus exercise mice (n=7) (**Panel A**). **Panel B** shows the IL-6 levels in the interscapular BAT for no-exercise (n=6) and exercise mice (n=7), when higher doses of total proteins were used in the Western Blot analyses – instead of 30 µg, we used 60 and 80 µg, in independent membranes. The upper band shows the IL-6 levels when a total protein mass of 60 µg was used, whereas the lower band show IL-6 levels when a total protein mass of 80 µg/mL was used.

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STUDY VI

Association of objectively measured physical activity with brown adipose tissue volume and activity in young adults

ABSTRACT

Human BAT has gained considerable attention as a potential therapeutic target for obesity and type 2 diabetes. However, whether physical activity (PA) might be an efficient stimulus to activate and recruit brown adipose tissue (BAT), as well as to induce browning of specific adipose tissue depots, remains to be ascertained. We aimed to examine whether objectively measured PA levels were associated with BAT volume and activity, and the expression of brown and beige markers in the subcutaneous white adipose tissue (sWAT), in young sedentary adults. We additionally examined the association of PA levels with the skeletal muscles activity.

A total of 130 young healthy and sedentary adults (67% women, age: 21.9±2.1 years old, body mass index: 25±4.8 kg/m^2) participated in this cross-sectional study. PA was objectively measured with a wrist-worn accelerometer (GT3X+, Actigraph, Pensacola, FL) for 7 consecutive days. Age-specific cut points were applied to classify wrist accelerations into sedentary time and different PA intensities (i.e., light, moderate, vigorous, moderate-vigorous). The participants underwent 2 hours of a personalized cold exposure to determine the cold-induced BAT volume and activity and the skeletal muscles activity by means of a ^{18}F-fluorodeoxyglucose positron emission tomography combined with a computed tomography scan. In an independent day, a biopsy was performed in the abdominal sWAT, and the relative expression of brown and beige markers was assessed by means of real time quantitative polymerase chain reaction.

Objectively measured PA intensity levels were neither associated with BAT volume and activity nor with the skeletal muscles activity (all P>0.05). The results remained after adjusting for sex, waking time, and environmental temperature. Similarly, no association of PA intensity levels was found with the expression of brown and beige markers in the sWAT (all P>0.05).

Although PA plays an important role in the prevention of obesity and related comorbidities, it seems that other physiological mechanisms rather than brown adipocyte activation or recruitment, or browning of the sWAT, might moderate its beneficial metabolic effects in young sedentary adults.

INTRODUCTION

During the last decade, several studies have confirmed the presence of active brown adipose tissue (BAT) in adult humans[1–4]. Human BAT is a unique thermogenic organ, able to dissipate energy in the form of heat through the action of the uncoupling protein 1[5]. BAT has gained considerable attention as a potential therapeutic target for obesity and type 2 diabetes[6]. In fact, BAT is inversely associated with body mass index and adiposity[2–4], and it seems to contribute to the regulation of glucose and lipid metabolism[7,8] as well as to the improvement of peripheral insulin sensitivity[9].

Both cold exposure and the use of specific β-adrenergic agonists and bile acids are the main activators of human BAT. Nevertheless, their use as long-term and translatable approaches to activate BAT remains unclear[6], being necessary to focus on the search of new strategies. Exercise has been previously proposed as another potential strategy that might activate and recruit human BAT[10,11]. Whereas data from human studies are still scarce and contradictory, findings from murine studies suggest a potential regulator effect of exercise on BAT metabolism[10,12]. Furthermore, there is an emerging body of novel adrenergic-independent activators, such as cardiac natriuretic peptides, irisin, interleukin-6, lactate, β-aminoisobutyric acid, meteorin-like, and fibroblast growth factor 21 (among others), that are sensitive to exercise and could therefore potentially influence the BAT function[10,11,13,14].

To date, findings from case control studies[15,16] have shown that trained individuals have lower cold-induced BAT volume and activity than sedentary or untrained individuals, as estimated by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography combined with computed tomography (PET/CT). Similarly, a pilot study[17] incidentally found lower BAT activity in trained versus sedentary control participants. Recently, Motiani et al.[18] also showed that 2 weeks of exercise (cycling) decreased insulin-stimulated BAT ^{18}F-FDG uptake in participants with active BAT prior to the intervention. Taken together[12,14–18], it seems that exercise is associated with the downregulation of BAT glucose metabolism, although further studies are needed to better understand the exercise-induced adaptations of human BAT.

Less attention has been paid, however, to the role of modifiable lifestyle behaviours such as physical activity (PA) on the activation and recruitment of human BAT. PA

comprises any bodily movement produced by the skeletal muscles which leads to an increase in energy expenditure above rest (i.e., 1.5 metabolic equivalents). PA is strongly related to the regulation of adipose tissue physiology[19] and plays an important role in the prevention of chronic diseases, such as obesity and type 2 diabetes[20,21]. Thus, it might be plausible that PA exerts its protective effects against these chronic diseases through the enhancement of the BAT function[11,22]. Only one study[23] in cancer patients has examined the association between self-reported PA and BAT activity estimated by ^{18}F-FDG-PET/CT, and showed that those participants who reported to be more active had higher levels of BAT activity. Findings of this study are, however, limited due to the fact that it presented several methodological problems that hamper the generalization of its results[24].

Similarly, the role of physical activity in the apparition of brown-like adipocytes within white adipose tissue remains unexplored. Until now, it has been shown that chronic exercise has little or no effect of on the expression of selected browning genes in the abdominal subcutaneous white adipose tissue (sWAT)[16,25-27]. However, only a study carried out by the of group Dinas et al.[28] has examined the role of PA on browning of sWAT. In this study, they showed that healthy adult men who reported moderate intensity PA levels had higher expression of browning markers (PGC-1α, PPARα, or PPARγ) that those who reported low intensity PA levels, in the abdominal sWAT[29]. Nevertheless, this study also presents several limitations, and whether objectively measured PA levels are related to the expression of brown and beige genes in the human sWAT needs to be further addressed.

On the other hand, it has been suggested that the skeletal muscle may have a key role in non-shivering thermogenesis[30-32]. Since the skeletal muscle is the most abundant tissue in the adult human body, slight changes in its thermogenic activity might induce important changes in whole body energy metabolism. Hence, to know whether PA is associated with the skeletal muscle cold-induced glucose uptake is of great interest.

This study aimed, therefore, to examine whether objectively measured PA levels are associated with BAT and the skeletal muscle ^{18}F-FDG uptake, as well as with the expression of brown and beige genes in the sWAT, in young healthy and sedentary

adults. All measurements were taken following the most updated methodology and international recommendations[33,34].

MATERIAL AND METHODS

Research design and participants

This cross-sectional study was carried out under the framework of the ACTIBATE study[35] (ClinicalTrials.gov, ID: NCT02365129). A total of 144 young healthy and sedentary adults initially took part in this study, from which 14 participants were excluded due to problems in data collection or analysis. Hence, a final sample of 130 participants (67% women) were included in the current analyses (see Flow-chart of the participants, **Figure S1[36]**). The participants were recruited through advertisements in electronic media and leaflets. All assessments were performed in Granada (south of Spain) during the months of October, November, and December 2015 and 2016. The inclusion criteria were being 18-25 years old, being sedentary (participants reported to practice <20 min of moderate-vigorous physical activity on <3 days/week), not smoking or taking any medication, having had a stable body weight in the last 3 months (changes <3 kg), and not presenting any cardiometabolic disease (e.g.: hypertension or diabetes).

The study protocol and the written informed consent were performed in accordance with the Declaration of Helsinki (revision of 2013). The study was approved by the Ethics Committee on Human Research of the University of Granada (nº 924) and of the "Servicio Andaluz de Salud" (Centro de Granada, CEI-Granada).

Procedures

PA and sedentary time

PA levels and sedentary time were objectively measured with a wrist-worn accelerometer (ActiGraph GT3X+, Pensacola, FL, US) for 7 consecutive days (24 hours/day)[35]. The participants came to the research center, and they were given detailed information on how to wear the accelerometer. They were also asked to remove it only during water activities such as bathing or swimming.

The accelerometers were initialized to store raw accelerations at a sampling frequency of 100 Hz[37]. The raw accelerations were exported and converted to ".csv" format using ActiLife v. 6.13.3 software (ActiGraph, Pensacola, FL, US). The raw ".csv" files were

then processed using the GGIR package (v. 1.5-12, https://cran.r-project.org/web/packages/GGIR/) in R (v. 3.1.2, https://www.cran.r-project.org/).

The processing methods of GGIR included: i) Auto-calibration of the data according to the local gravity[38]; ii) Calculation of the Euclidean Norm Minus One (ENMO) as √(x^2+ y^2+ z^2) -1G (where 1G ~ 9.8 m/s^2) with negative values rounded to zero; iii) Detection of the non-wear time based on the raw acceleration of the three axes, briefly, each 15-min block was classified as non-wear time if the standard deviation of 2 out of the 3 axes was lower than 13 mG during the surrounding 60-min moving window or if the value range for 2 out of the 3 axes was lower than 50 mG; iv) Detection of sustained abnormal high accelerations, i.e., higher than 5.5 G during at least 15 minutes, related to malfunctioning of the accelerometers; v) Imputation of detected non-wear time and abnormal high accelerations by means of the acceleration for the rest of the recording period during the same time interval than the affected windows; vi) Identification of waking and sleeping hours with an automatized algorithm guided by the participants' diary reports[39]; and vii) Estimation of the time spent in sedentary behaviour and in different PA intensities [light (LPA), moderate (MPA), vigorous (VPA), and moderate-vigorous (MVPA)] using age-specific cut-points for ENMO[40,41].

Additionally, we calculated the time spent in moderate-vigorous PA in bouts of ≥ 10 minutes ($MVPA_{10min}$) with a drop-down tolerance of 2 minutes. We used the mean ENMO (mG) during waking time as an overall indicator of the PA level. Only the participants wearing the accelerometers for ≥16 hours/day during at least 4 days (including at least 1 weekend day) were included in the analyses.

Simultaneously, we attached a hip-worn accelerometer to the participants and replicated the analyses using the previously published hip-based cut-points by Hildebrand et al.[40,41] to classify hip ENMO into sedentary time or LPA, MPA, VPA and MVPA.

Personalized cold exposure and ^{18}F-FDG-PET/CT acquisition

The participants underwent a personalized cold exposure, and then BAT, skeletal muscles, and descending aorta glucose uptake were assessed by a ^{18}F-FDG-PET/CT scan (Siemens Biograph 16 PET/CT, Siemens, Germany)[42]. The cold-induced ^{18}F-FDG-PET/CT acquisition was performed according to the current methodological

recommendations[34]. Briefly, the participants arrived at the lab and confirmed that they had met the pre-study conditions: arriving in fasting state (at least 6 hours), having slept as usual, having refrained from any moderate (within 24 hours) or vigorous (within 48 hours) physical activity, and not having consumed any alcoholic or stimulant beverages (within 6 hours) or drugs which could have affected the peripheral circulation (within 24 hours). Then, they voided their bladders and dressed in standardized clothes. The participants stayed 30 minutes in a warm room (22-23°C) in order to acclimatize. After that, the participants entered a cold room (19.5-20°C) and wore a temperature-controlled water circulation cooling vest (Polar Products Inc., Ohio, USA) for 60 minutes set at ~4°C above their shivering threshold.

The shivering threshold was defined as the water temperature at which shivering onset was self-reported by the participants and visually determined by the evaluators in a gradual cooling protocol performed 48-72 hours prior to the PET/CT scan. After 60 minutes of personalized cold exposure we injected a bolus of ^{18}F-FDG (180.6 ± 5.8 MBq = 2.9 MBq / kg) and raised the water temperature of the vest ~1°C for the last 60 minutes to avoid shivering. To note is that the water temperature of the vest was increased an additional ~1°C when participants reported shivering. After 2 hours of personalized cold exposure, the participants went into the PET/CT scan. For the CT acquisition, a peak kilovoltage of 120 was applied, and a scan time of 6 min per bed position was set for the PET acquisition. In total, 2 bed positions were scanned, from *atlas vertebrae* to *thoracic vertebrae 6*[42].

^{18}F-FDG-PET/CT analysis

PET/CT scans were analyzed using the software based on Beth Israel plugin for FIJI http://sourceforge.net/projects/bifijiplugins/[43] by BMT. The analyses were conducted under the supervision of a nuclear medicine physician.

We calculated the standardized uptake value (SUV) as [^{18}F-FDG uptake (kBq/ml) / (injected dose [kBq] / patient weight [g])]. Firstly, we aimed to quantify BAT glucose uptake. With this purpose, we outlined 6 regions of interest (ROIs), from *atlas vertebrae* to *thoracic vertebrae* 4, using a 3D-axial technique[44]. These ROIs comprised the supraclavicular, laterocervical, paravertebral, and mediastinal regions. Within these ROIs, a SUV threshold for a voxel to be considered BAT was computed as SUV ≥ [1.2 / (lean body mass/body mass)], and a fixed range of Hounsfield Units (HU, -190 to

-10) was applied[34]. Then we calculated BAT volume as the sum of the volume in ml for each ROI; $SUV_{mean,}$ as the weighted average of SUV_{mean} derived from each ROI; and SUV_{peak} as the highest average SUV in a 1 ml spherical volume over all ROIs[34,45]. The PET/CT scans were visually and carefully examined to detect ^{18}F-FDG uptake in BAT-specific depots. For further analyses, we calculated BAT parameters using different combinations of SUV thresholds and HU ranges: i) SUV: 2, HU: -250 to -50; ii) SUV: 1.5, HU: -180 to -10.

We outlined 1 single slice-ROIs to determine the ^{18}F-FDG-uptake in the *cervical*, *scalene, longus colli, paravertebral, subscapular, sternocleidomastoid, supraspinous, trapezius, deltoid, pectoralis major, and triceps braquis* muscles from both the right and left side of the body. Then, we calculated an average of the SUV_{peak} in all muscles (from both the left and right side of the body). The skeletal muscle was distinguished from the adipose tissue by means of HU, in which the skeletal muscle was considered to lie between 10 and 100 HU. We additionally drew a 1 slice-ROI in the descending aorta (reference tissue) at the height of the *thoracic vertebrae 4*, and we obtained its SUV_{peak}. Finally, for confirmatory analyses, we computed BAT SUV_{mean} and SUV_{peak} as well as all muscles SUV_{peak} as a product of % lean body mass (SUV_{LBM}) as recently proposed[44].

Biopsy procedures

Biopsies were collected in an independent day that participants attended the research centre. All participants were instructed to come in fasting state (for 10 hours). Two expert surgeons performed the biopsies following the Bergström´s technique, as described elsewhere[46], using 2% mepivacaine as an analgesic agent. A biopsy from the abdominal subcutaneous white adipose tissue – approximately 2-3 cm to the side of the umbilicus - was performed, obtaining approximately 100 mg of white adipose tissue. Immediately after removal, tissue samples for RNA analysis were immediately flash frozen in liquid nitrogen, and store at -80ºC. All biopsies were normally performed within the same time frame (9:00-12:00).

Brown and beige gene expression in the abdominal subcutaneous white adipose tissue

RNA Isolation from adipose tissue biopsies was performed with Quiazol lysis reagent, using the RNeasy Lipid Tissue minikit (QUIAGEN, Hilden, Germany), and according to the manufacturer's recommendations. Once RNA was purified, it was dissolved in

nuclease-free water and quantified using fluorescent dyes (Qubit, Invitrogen, Thermo Fisher). Total RNA was reverse-transcribed using the iScript Reverse Transcription Supermix for qRT-PCR (Biorad, California, USA). cDNA samples were loaded (4 ng/well) in duplicates and quantitative real-time polymerase chain reaction (qrt-PCR) was performed using an ABI ViiA 7 Real-Time PCR system, 384 well block (Applied Biosystems, California, USA). All procedures were performed according to the manufacturer's protocol. Relative quantification was conducted by - SYBR- green fluorescent dye (Applied Biosystems, California, USA).

Target mRNAs (i.e., CIDEA, UCP1 and TBX1 mRNAs) were normalized to LRP10 (LDL receptor related protein 10), and relative expression (fold-change) was calculated with the $\Delta\ \Delta C_T$ method. Primers sequences of these genes can be found in Supplementary Material.

Body composition

We measured the participant's height and weight with a SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Lean mass and body fat mass were measured by Dual Energy X-ray Absorptiometry (HOLOGIC, Discovery Wi).

Environmental temperature

A clear link has been reported between the environmental temperature to which participants are exposed and the prevalence, volume, and activity of BAT estimated by ^{18}F-FDG-PET/CT[3,47]. Given the fact that the assessments were carried out throughout the months of October, November, and December 2015 and 2016, it is relevant to consider the confounding effect of the environmental temperature on BAT volume and activity. We obtained the local air temperature data of every hour of the entire study period from the Spanish State Meteorological Agency (AEMET). The hourly-obtained air temperature data were used to calculate the mean daily environmental temperature for the dates when the participants wore the accelerometers (which normally started approximately 1-2 weeks before the PET-CT scan, and lasted 7 days).

Statistical analysis

Descriptive data are reported as mean and standard deviation. There was no significant interaction between the effects of sex, sedentary time, or PA levels with BAT activity (P>0.05). We only found a significant interaction between the effects of sex and $MVPA_{10min}$ on BAT volume (t=2.56, P=0.012). Therefore, the analyses were

done for men and women together. Pearson correlations were firstly conducted to examine the association of the time spent in sedentary behaviour and different PA intensities with BAT volume and activity (SUV_{mean} and SUV_{peak}). Subsequently, multiple linear regressions (enter method) were performed to examine this relationship after adjusting for potential confounders. Model 1 was adjusted for sex and waking time; and Model 2 was adjusted for sex, waking time, and environmental temperature. Pearson correlations were also conducted to examine the association of the time spent in sedentary behaviour and different PA intensities with the ^{18}F-FDG uptake (SUV_{peak}) by the skeletal muscles and the descending aorta. As secondary analyses, we also conducted person correlations to examine the relationship of the time spent in sedentary behaviour and different PA intensities with the expression of brown and beige markers in the sWAT. Partial correlations were also performed to examine this relationship after adjusting for potential confounders (determined by exploratory correlations and following a theoretical basis). Of note, all variables related to the expression of brown and beige markers in the sWAT were transformed (log10) to make their distributions closer to the normal distribution. The level of significance was set at $P<0.05$. Statistical trends were reported as $P>0.05$ and ≤ 0.1. The statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS version 24, Inc. Chicago, IL, USA).

RESULTS

Table 1 shows the descriptive characteristics of the study participants. The participants wore the accelerometer an average of 23.1±0.5 hours/day. Sedentary time accounted for 79% of their waking time.

	All (n = 130)	**Women** (n = 87)	**Men** (n = 43)
Age (years)	21.9 (2.1)	21.7 (2.1)	22.2 (2.2)
Professional status, n (%)*			
Student	65 (51)	44 (51)	21 (51)
Unemployed	43 (34)	32 (37)	11 (27)
Other professional activities	19 (15)	10 (12)	9 (22)
Body composition			
BMI (kg/m^2)	25 (4.8)	23.8 (3.9)	27.5 (5.5)
LMI (kg/m^2)	14.6 (2.5)	13.3 (1.4)	17.2 (2.1)
FMI (kg/m^2)	9 (3)	9.2 (2.7)	8.7 (3.6)
Body fat mass (%)	36.2 (7.3)	38.6 (5.9)	31.3 (7.6)
^{18}F-FDG uptake parameters			
BAT Volume (ml)	70 (58.7)	64.2 (52.7)	81.7 (68.6)
BAT SUV_{mean} (g/ml)	3.8 (2)	4 (2.1)	3.4 (1.5)
BAT SUV_{peak} (g/ml)	11.4 (8.4)	11.9 (8.7)	10.3 (7.7)
All muscles SUV_{peak} (g/ml)	0.8 (0.2)	0.8 (0.2)	0.8 (0.2)
Descending aorta SUV_{peak} (g/ml)	1.6 (0.3)	1.5 (0.3)	1.6 (0.4)
Sedentary behaviour and PA			
Valid days (days)	6.8 (0.6)	6.8 (0.5)	6.7 (0.6)
Wear time (hours/day)	23.1 (0.5)	23.2 (0.5)	23 (0.5)
Waking time (hours/day)	15.5 (0.8)	15.5 (0.8)	15.4 (0.9)
Sedentary time (min/day)	731 (59.7)	728.6 (54.2)	735.9 (70)
LPA (min/day)	113.7 (27)	118.2 (24.8)	104.8 (29.3)
MPA (min/day)	81.2 (28.8)	82.2 (27.4)	79.1 (31.8)
VPA (min/day)	2.7 (3.3)	2.7 (3.4)	2.6 (3.3)
MVPA (min/day)	83.9 (30.5)	85 (29.2)	81.7 (33.2)
$MVPA_{10min}$ (min/day)	21.6 (18.9)	21 (19.2)	22.8 (18.6)
Overall PA (ENMO, mG/5s)	31.9 (8.3)	32.4 (7.8)	30.9 (9.3)

Table 1. Characteristics of the participants. Continuous variables are presented as mean (standard deviation) and categorical variables as number (percentage). *For the variables related to the professional status of the participants, a total sample size of 127 was included in the descriptive analyses since 3 cases were missed. BAT: brown adipose tissue, ENMO: Euclidean norm minus one, FMI: fat mass index, LMI: lean mass index, LPA: light physical activity, MPA: moderate physical activity, MVPA: moderate-vigorous physical activity, $MVPA_{10min}$, moderate-vigorous physical activity in bouts of 10 minutes, SUV: standardized uptake values, VPA: vigorous physical activity.

PA levels are not associated with BAT and skeletal muscle ^{18}F-FDG uptake

There was no association between the time spent in different PA intensities and BAT volume (**Figure 1A**, all P>0.05) and activity (SUV_{mean} and SUV_{peak}**, Figures 1B and 1C**, respectively, all P>0.05).

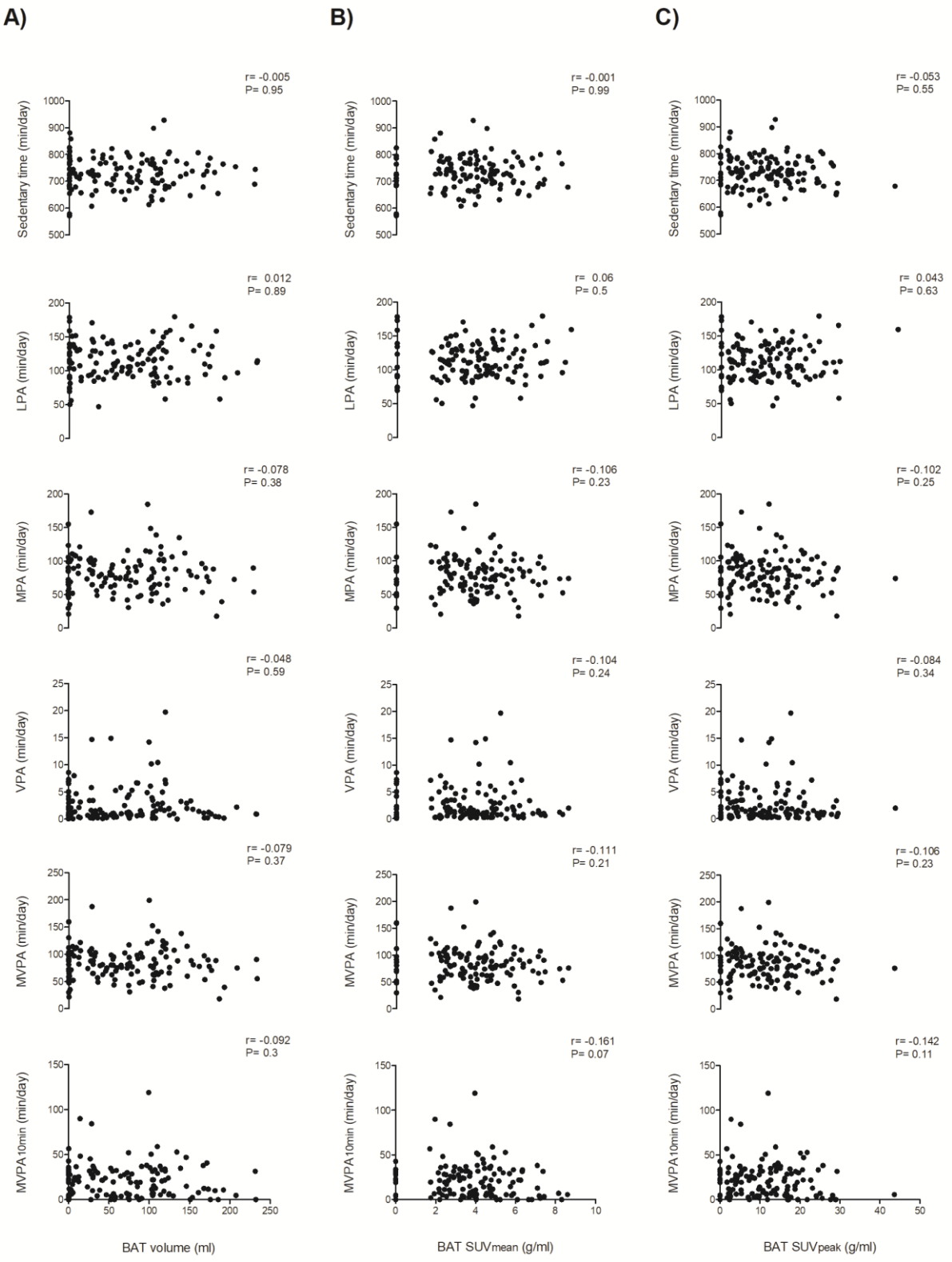

Figure 1. Association of the time spent in sedentary behaviour and in different physical activity (PA) intensities with brown adipose tissue (BAT) volume and activity [standardized uptake value (SUV) mean and peak] in young sedentary adults, n =130. Pearson correlations were performed to examine the association of the time in sedentary behaviour and in different physical activity (PA) intensities with BAT volume (**Panel A)**, SUV_{mean} **(Panel B)**, and SUV_{peak} **(Panel C)**. LPA: light physical activity, MPA: moderate physical activity, MVPA: moderate-vigorous physical activity, $MVPA_{10min}$, moderate-vigorous physical activity in bouts of 10 minutes, VPA: vigorous physical activity.

Linear regression analyses adjusted for sex and waking time (Model 1) and for sex, waking time, and environmental temperature (Model 2) showed that the association of the time spent in different PA intensities with BAT volume and activity (SUV_{mean} and SUV_{peak}) was not significant (all P>0.05, see **Table 2**). Only $MVPA_{10min}$ showed an inverse borderline association with BAT SUV_{mean} when it was not adjusted for any covariable (P=0.07, **see Figure 1B)** or in Model 2 (P=0.09, **Table 2)**. The results did not change when we included body, lean, or fat mass index as well as body fat percentage, in any statistical model, or when we normalized SUV to lean body mass (SUV_{LBM}), instead of total body mass (SUV_{BM}), to calculate BAT SUV_{mean} and SUV_{peak} (data not shown). Similarly, Pearson correlations showed that PA levels were not associated with all skeletal muscles and descending aorta activity (SUV_{peak}) (**Figures 2 and S2**[31], respectively; all P>0.05). A lack of association was also observed between overall PA and BAT volume and activity (SUV_{mean} and SUV_{peak}) and all skeletal muscles and descending aorta activity (SUV_{peak}) (All P>0.05, data not shown).

Sedentary time is not associated with BAT and skeletal muscle ^{18}F-FDG uptake

Sedentary time was associated with neither BAT volume and activity (Figure 1), nor all skeletal muscles and the descending aorta activity (SUV_{peak}) (**Figures 2 and S2**[36], respectively; all P>0.05). The results replicated when we adjusted the analyses for Model 1 and Model 2 (data not shown).

Table 2. Association of the time spent in sedentary behaviour and in different physical activity (PA) intensities with brown adipose tissue (BAT) volume and activity [standardized uptake value (SUV) mean and peak], skeletal muscles activity (SUV_{peak}), and descending aorta (SUV_{peak}) in young sedentary adults, n = 130.

	BAT volume			**BAT SUV_{mean}**			**BAT SUV_{peak}**			**All muscles SUV_{peak}**			**Descending aorta SUV_{peak}**		
	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P
Sedentary time (min/day)															
Model 1	0.011	-0.002	0.92	0.002	0.008	0.59	0.004	-0.001	0.79	0	-0.007	0.35	0	0.005	0.85
Model 2	-0.002	0.094	0.99	0.002	0.104	0.66	0.002	0.118	0.89	0	-0.004	0.33	0	-0.002	0.84
LPA (min/day)															
Model 1	0.144	0.002	0.49	0.003	0.007	0.64	0.02	0.002	0.49	0.001	-0.004	0.28	0	0.006	0.81
Model 2	0.118	0.096	0.55	0.002	0.104	0.72	0.016	0.12	0.56	0.001	-0.003	0.3	0	-0.002	0.8
MPA (min/day)															
Model 1	-0.135	0.002	0.46	-0.007	0.017	0.23	-0.026	0.006	0.33	0	-0.009	0.45	0	0.007	0.65
Model 2	-0.088	0.095	0.62	-0.006	0.11	0.32	-0.018	0.121	0.46	0.001	-0.006	0.4	-0.001	-0.001	0.63
VPA (min/day)															
Model 1	-0.72	0	0.65	-0.061	0.016	0.24	-0.185	0.004	0.41	-0.005	-0.006	0.33	-0.006	0.009	0.49
Model 2	-0.111	0.094	0.94	-0.034	0.106	0.5	-0.055	0.118	0.8	-0.004	-0.006	0.41	-0.007	0.002	0.47
MVPA (min/day)															
Model 1	-0.13	0.002	0.46	-0.007	0.018	0.2	-0.025	0.007	0.31	0	-0.011	0.54	-0.001	0.007	0.61
Model 2	-0.078	0.095	0.64	-0.006	0.11	0.31	-0.017	0.121	0.47	0	-0.007	0.48	-0.001	0	0.6
$MVPA_{10min}$ (min/day)															
Model 1	-0.299	0.007	0.28	-0.016	0.029	0.09	-0.057	0.016	0.14	-0.001	-0.011	0.53	0	0.006	0.8
Model 2	-0.188	0.097	0.47	-0.012	0.116	0.17	-0.04	0.126	0.28	0	-0.009	0.62	0	-0.002	0.78

Linear regression analyses were performed, adjusting for sex and waking time (Model 1); and for sex, waking time and environmental temperature (Model 2). Non-standardized β coefficient, adjusted R squared and P-value are provided. Statistically significant values are shown in bold. LPA: light physical activity, MPA: moderate physical activity, MVPA: moderate-vigorous physical activity, $MVPA_{10min}$, moderate-vigorous physical activity in bouts of 10 minutes, VPA: vigorous physical activity.

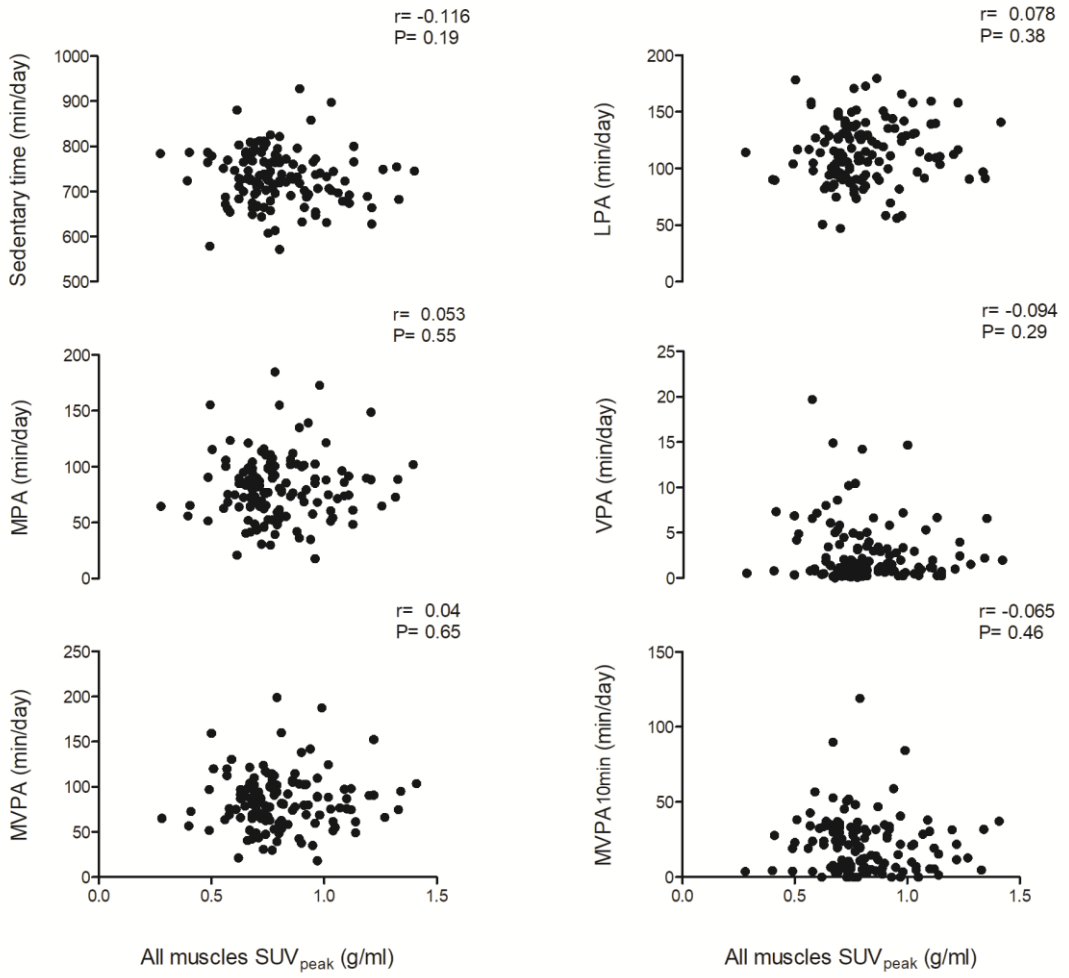

Figure 2. Association of the time spent in sedentary behaviour and in different physical activity (PA) intensities with the skeletal muscles activity [standardized uptake value (SUV) peak] in young sedentary adults, n =130. Pearson correlations were performed. LPA: light physical activity, MPA: moderate physical activity, MVPA: moderate-vigorous physical activity, $MVPA_{10min}$, moderate-vigorous physical activity in bouts of 10 minutes, VPA: vigorous physical activity.

The results remained unchanged when we repeated the main analyses using the accelerometer placed on the hip, the evaluation wave (moment in which the participants were evaluated) instead of the environmental temperature, or when we included the physical fitness, menstrual cycle of female participants (in the PET/CT evaluation) or professional status as potential confounders. Moreover, we performed the analyses using different combinations of SUV thresholds and HU ranges to quantify BAT volume and activity [i) SUV: 2, HU: -250 to -50; ii) SUV: 1.5, HU: -180 to -10] and all the findings persisted (data not shown).

MPA, MVPA and $MVPA_{10min}$ were significantly associated with cardiorespiratory fitness (all r=0.22, all P≤0.02). Moreover, $MVPA_{10min}$ was inversely associated with body fat percentage (r=-0.2, P=0.02) (**Table S1**)[36].

As secondary analyses we also examined whether the time spend in sedentary behaviour and different PA intensities was related to the expression of brown and beige genes in the SWAT. **Figure 3** shows the average C_T detected by qRT-PCR for the target genes (**Panel A)**, as well as the number of samples in which the reference or brown and beige genes could be detected **(Panel B).** UCP1 and TBX1 showed a higher average CT, translating to low expression.

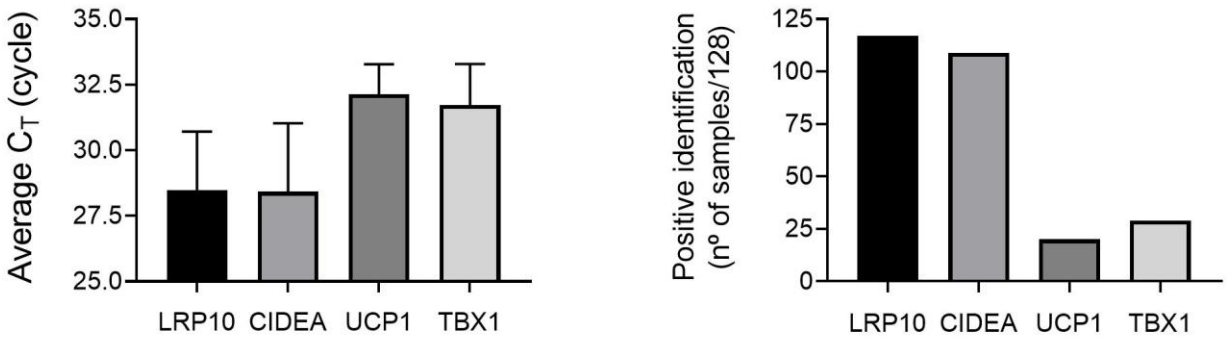

Figure 3. Expression of brown and beige genes in the abdominal subcutaneous white adipose tissue in young adults. **Panel A:** Average C_T detected by qRT-PCR. UCP1 and TBX1 genes have high C_T values, translating to low expression. LRP10 was used as the reference (i.e., normalizing) gene. **Panel B:** positive identification of the reference and and brown/beige genes in the subcutaneous WAT, as determined by qRT-Pcr. The total sample size (number of biopsies obtained) were 128.

When examining the relationship of the time spent in sedentary behaviour and different PA intensities with the relative expression of brown and beige genes in the sWAT, no significant relationship was found **(Figure 4).** Similar results were observed when the analyses were independently adjusted for sex, fat mass index, or the day in which the evaluation was performed (an indicator of seasonality) **(see Table 3).** The relationship of the expression of brown and beige markers in the sWAT with body composition parameters is shown in the Supplementary Material (see **Table S1).** There was no significant association between the relative expression of UCP1 and TBX1 with body composition parameters; however, the expression of CIDEA was inversely related to BMI, fat-free mass index, body fat percentage, fat mas index and visceral adipose tissue (VAT) mass (r=-0.463, -0.314, -0.461, -0.494, -0.389, all P≤0.001).

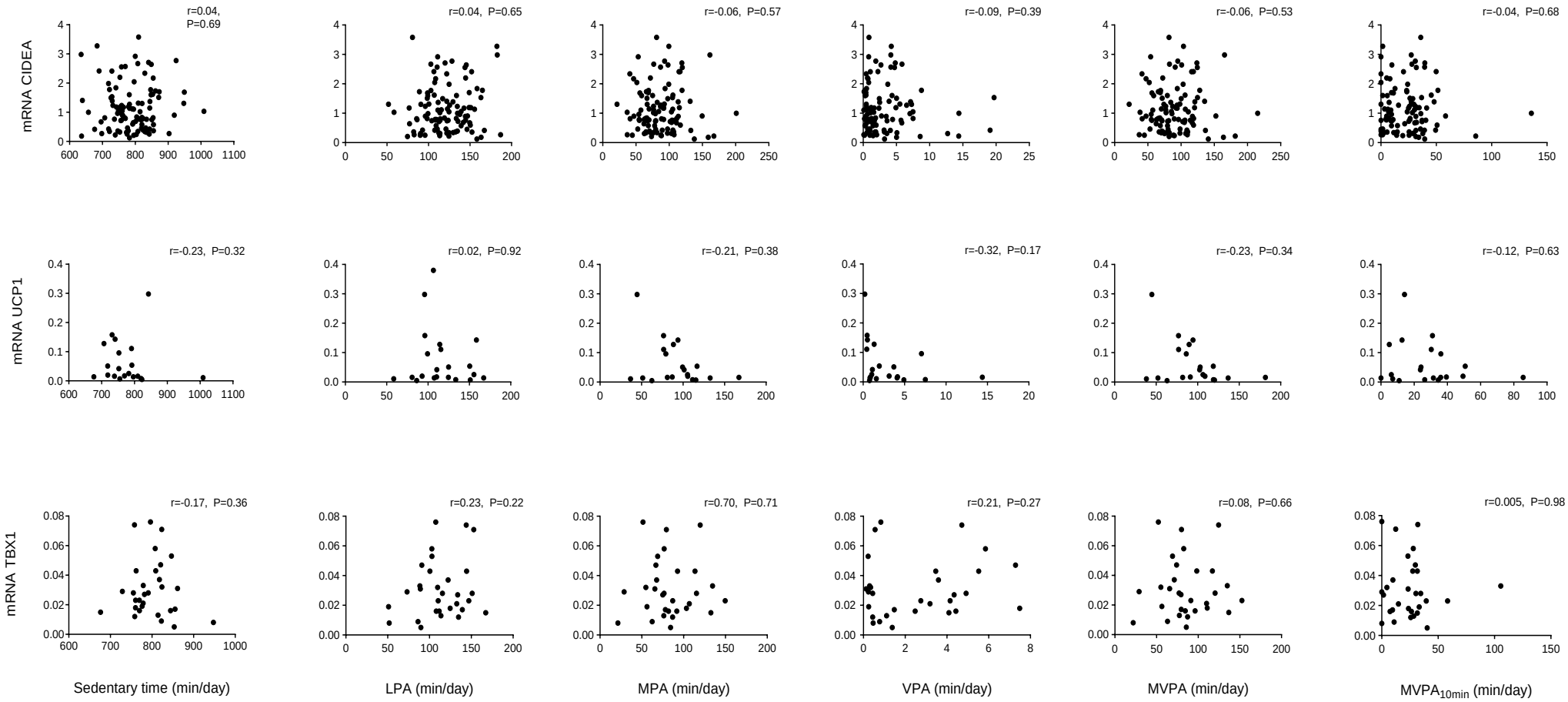

Figure 4. Association of objectively measured PA with the expression of brown (CIDEA, UCP1) and beige (TBX1) genes in the abdominal subcutaneous white adipose tissue, in young adults. Sample size for the relationship between accelerometry measures and gene expression was 103 for CIDEA, 20 for UCP1, and 30 for TBX1. The standard curve with LRP10 as the normalizing gene was used to determine relative gene expression levels. All variables related to gene expression were transformed. No significant associations were found (all P>0.05).

	Sedentary time	LPA	MPA	VPA	MVPA	$MVPA_{10min}$
Model 1 (adjusted for sex)						
mRNA CIDEA	0.026	0.067	-0.045	-0.079	-0.052	-0.038
mRNA UCP1	-0.230	0.019	-0.214	-0.322	-0.232	-0.117
mRNA TBX1	-0.166	0.222	0.063	0.202	0.076	0.002
Model 2 (adjusted for fat mass index)						
mRNA CIDEA	0.096	0.077	-0.078	-0.155	-0.091	-0.131
mRNA UCP1	-0.251	0.017	-0.208	-0.309	-0.224	-0.094
mRNA TBX1	-0.158	0.248	0.067	0.190	0.078	-0.030
Model 3 (adjusted for evaluation wave)						
mRNA CIDEA	0.041	0.045	-0.057	-0.091	-0.065	-0.044
mRNA UCP1	-0.240	0.025	-0.207	-0.307	-0.223	-0.109
mRNA TBX1	-0.182	0.235	0.078	0.232	0.094	0.015

Table 3. Association of objectively measured PA with brown (CIDEA, UCP1) and beige (TBX1) genes expression in the abdominal subcutaneous white adipose tissue in young adults, after adjusting for potential confounders. Partial correlations were performed to examine the association of physical activity parameters with the gene expression of CIDEA (Model 1, n=102; Model 2, n=101; Model 3, n=99), UCP1 (n=19 for all models), and TBX1 (Model 1, n=29; Model 2, n=28; Model 3, n=27). The standard curve with LRP10 as the normalizing gene was used to determine relative gene expression levels. All variables related to gene expression were transformed. ***P<0.001. No significant associations were found for UCP1 and TBX1 relative gene expression. BMI: body mass index, FFMI: fat free mas index, FMI: fat mass index, VAT mass: visceral adipose tissue.

DISCUSSION

The present study shows, for the first time, that objectively measured free-living PA levels are not associated with BAT volume and activity estimated by ^{18}F-FDG uptake in young sedentary adults. Similarly, PA levels are not associated with the skeletal muscles activity. These findings persisted after accounting for the confounding effect of sex, waking time, and environmental temperature. Of note is that the measurement of PA[33,37] and quantification of BAT[34] were performed strictly following the most updated methodological recommendations. These results shed light on the possible role of PA as a regulator of human BAT.

Our findings do not concur with previous observational evidence[23], which showed that self-reported PA was positively associated with BAT activity, and that there were significant differences in BAT activity across low/moderate/high levels of PA (P<0.05). These discrepancies could be partially explained by several methodological aspects[24]. Firstly, the sample of Dinas et al.[23] study was composed by 40 participants (65% men) with cancer, and it is possible that tumor cells competed with brown and beige adipocytes for ^{18}F-FDG uptake. In addition, they assessed

habitual PA with a questionnaire, which could be biased by social desirability and cognitive challenge. Objective methods, such as accelerometry, are more accurate and reliable to monitor PA levels[48]. To note also is that, in contrast with studies highlighting the need of a previous cooling protocol to induce BAT activation[34,49], Dinas et al.[23] performed the ^{18}F-FDG-PET/CT scan in warm conditions.

In contrast with the abovementioned observations, Vosselman et al.[16] reported that endurance-trained men had lower levels of ^{18}F-FDG uptake by BAT compared to sedentary controls, whereas no differences in abdominal sWAT browning markers expression between cohorts were observed. Similarly, Singhal et al.[15] showed that trained women presented lower levels of ^{18}F-FDG uptake by BAT compared to untrained-women, which concurred with another observational study conducted in 2 endurance athletes and 10 untrained individuals[17]. These findings together with those by Motiani et al.[18], who showed that a short term exercise (cycling) training decreased insulin-stimulated BAT ^{18}F-FDG uptake in those participants with active BAT, suggest that exercise downregulates human BAT ^{18}F-FDG uptake in adult humans, whereas PA levels are not associated to it in young sedentary adults. Despite the fact that both constructs (exercise and PA) are different[50], many of their metabolic and physiological responses are mediated by the contracting activity of the skeletal muscle[51,52]. Thus, a minimal dose in terms of duration and intensity of the contracting activity of the skeletal muscle might be needed to induce adaptations in human BAT. This fact would partially address why in previous observational studies only the trained participants (those performing exercise in a programmed, structured, and aimed-goal way) showed a decreased BAT glucose metabolism, whereas we observed a lack of association between PA and BAT function in our sedentary participants. In line with this, we observed that $MVPA_{10min}$, the component related to a more intense, repetitive, and structured PA, showed an inverse association with BAT SUV_{mean} in our sample. A study sample with participants more active or with less homogeneous PA levels might have unmasked an inverse relationship between PA levels and BAT function. Nevertheless, it is important to consider that the results from the previous observational studies may be influenced by the differences in PA or fitness in the participants.

The underlying mechanisms that could explain the lack of association between PA levels and BAT remain to be further explored. On the one hand, it is well known

that exercise generates heat and that it can increase the internal body temperature[53]. Consequently, it seems coherent that an hyperthermic stimulus, such as exercise, might abolish the thermogenic role of BAT in order to maintain the thermoregulatory homeostasis[12,14]. Exercise generates heat through contracting the skeletal muscle, and, consequently, it might be possible that BAT would not play a significant role when PA is performed. On the other hand, several studies[31,32] have suggested that the skeletal muscle may play a key role in non-shivering thermogenesis, by means of sarcolipin mediated sarcoplasmatic/endoplasmic reticulum calcium ATPases (SERCAs), especially in large mammals (such as humans), where BAT content is reduced in relation to body size. Hence, it could be speculated that the possible recruitment of a large skeletal muscle mass when performing PA avoids the need for BAT activation when humans are exposed to cold. This would be in line with the fact that the skeletal muscle seems to have a higher capacity than BAT to generate heat in humans[14,54,55]. To test this hypothesis, we examined whether PA levels were associated with cold-induced skeletal muscle ^{18}F-FDG uptake, but we did not find any significant association. No previous studies have examined this relationship, which precludes across study comparisons. However, Vosselman et al.[16] compared cold-induced skeletal muscle ^{18}F-FDG uptake in trained versus sedentary participants, and observed that it was similar in both groups, which supports our results. Taken together, these findings call into question whether other physiological mechanisms might be involved. In any case, it is important to consider that: i) a mild cold exposure might not be the best method to assess the skeletal muscle ^{18}F-FDG uptake, since it is likely to prompt a predominantly fat oxidative metabolism[30], and ii) only the skeletal muscles of the upper part of the body were analysed, and habitually trained muscles should be included.

Further studies are required to better understand the relationship between PA and BAT function, beyond glucose metabolism. PA induces different patterns of skeletal muscle contractive activity[52], that at the same time modulates the release of muscle-derived factors (myokines)[14,51,56]. These myokines are able to regulate physiological processes in other tissues[51,56], such as WAT (possibly inducing browning), and might also influence BAT. In addition, the secretion of other factors during PA (such as adipokynes, hepatokines, cardiac natriuretic peptides, and brain-derived factors) could also influence these tissues. A deeper understanding of the role of PA in

human BAT function could also provide a new insight into human energy balance. Increasing PA levels is normally related to a higher total energy expenditure (at least during the initial stages in sedentary people), reductions in adiposity, and changes in substrate metabolism preference[19,57]. As a consequence of these physiological changes, which could lead to a state of relative energy deficit as trained status or PA levels are increased[15], BAT may undergo adaptive reductions in order to favour a more efficient energy metabolism[22]. Finally, other possible factors should be considered, such as an increased BAT glycogen capacity (reducing the need of circulating ^{18}F-FDG uptake)[18] or a higher utilization of lipids by BAT (increased mitochondrial biogenesis, increased angiogenesis, changes in intermediate metabolites), with increased PA levels.

Whether browning - the apparition of brown-like adipocytes within WAT– can be induced by exercise in humans, especially in subcutaneous depots, also remains unknown[14]. Evidence has shown little or no effect of chronic exercise on the expression of selected browning genes in the abdominal sWAT[14,25,26,27]. Even less is known about the relationship between habitual PA levels and the browning of specific adipose tissue depots. Until now, only the group of Dinas et al. has reported that the expression of several browning formation markers is more highly expressed in the sWAT of healthy adult men displaying moderate rather than low PA levels[28]. Nevertheless, this study presents several limitations that hampers its interpretation. For instance, results cannot be generalized beyond men, they assessed PA with subjective methods, and they did not measure specific browning related genes (e.g., TBX1 or TMEM26). Contrary to them, we showed that objectively measured PA (at different intensities) was not related to the expression of brown/beige genes in the sWAT in young healthy adults, even when adjusted for potential confounders. Therefore, this relationship is still controversial and more research is needed. It is noteworthy that, UCP1 and TBX1 were scarcely detected in sWAT, indicating that browning in this depot is unlikely in humans, and that other depots should be explored[58].

A limitation to consider in the present study is its cross-sectional design, which prevents a causal interpretation of our results. It might be speculated the possibility of reverse causality, such that subjects with low or no BAT tend to move around to get

warm. This is a plausible option, since humans cope with cold through the combination of both physiological and behavioural mechanisms such as clothing, postural changes, temperature choice, or physical movement [48,53]. Nevertheless, it seems coherent that participants with low or no BAT might be more likely to rely on behavioural mechanisms such as clothing, postural changes, or the use of heating (in the modern-societies) when they are exposed to cold (at least during the initial stages)[59], rather than moving or exercising to get warm, which would be translated into a worse energy efficiency. Well-designed randomized controlled trials should be carried out in order to elucidate the role of PA (if any) in BAT function. In addition, we do not know whether our findings apply to older people or individuals presenting any metabolic disease (e.g. type 2 diabetes). The homogeneous PA levels of our sedentary sample might have also influenced the results (explaining the lack of a significant relationship between PA levels BAT function). Histological examinations across different adipose depots should also be performed to confirm whether objectively measured PA levels are associated with the recruitment of brown and beige adipocytes in sWAT. Of note is also that statistical trends might be a consequence of the repeated testing effects bias.

On the other hand, despite being the most extended technique, ^{18}F-FDG PET-CT presents some limitations which might not allow to accurately estimate cold-induced BAT metabolic activity, such as the fact that it is related to glucose metabolism, and fatty acids are the main substrate of brown adipocytes in humans[60]. Whether our findings will replicate when using other radiotracers such as ^{15}O-labeled oxygen, ^{11}C-acetate or ^{18}F-fluoro-6-thia-heptadecanoic acid (FTHA) to quantify BAT metabolism is not known.

In conclusion, we show that objectively measured PA levels are not associated with either BAT volume and activity or skeletal muscles activity in young healthy and sedentary adults. Although PA is well-known to have an important role in the prevention of chronic diseases, such as obesity and type 2 diabetes, it could be that other physiological mechanisms beyond brown adipocyte activation or recruitment, and browning of specific adipose tissue depots, may moderate its beneficial metabolic adaptions.

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SUPPLEMENTARY MATERIAL

Primers sequences

Primers sequences for these genes were designed and tested in tissue samples from 2 different cohorts. Intron-spanning primers used for SYBR-green qPCR are as follows: LRPA forward primer 5'-TGTAAAACGACGGCCAGT-3' and reverse primer 5'- 5' CAGGAAACAGCTATGACC-3', CIDEA: forward primer 5'- GACTCAAGGGCCTGCTGA-3' and reverse primer 5'- GAAACTGTCCCGTCACCT-3'; UCP1: forward primer 5'- AGGTCCAAGGTGAATGCCC -3' and reverse primer 5'- GCGGTGATTGTTCCCAGGA-3', TBX1: forward primer 5'- CGCAGTGGATGAAGCAAATCGTG-3' and reverse primer 5'- TTTGCGTGGGTCCACATAGACC-3'.

Table S1. Association of body composition parameters with brown (CIDEA, UCP1) and beige (TBX1) gene expression in the abdominal subcutaneous white adipose tissue in young adults, after adjusting for sex.

	BMI	FFMI	Body fat %	FMI	VAT mass
mRNA CIDEA	**-0.463*** **	**-0.314*** **	**-0.461*** **	**-0.494*** **	**-0.389*** **
mRNA UCP1	0.118	0.019	0.214	0.164	0.118
mRNA TBX1	-0.244	-0.203	-0.274	-0.242	-0.348

Partial correlations were performed to examine the association of body composition parameters with the gene expression of CIDEA (n= 101), UCP1 (n=19), and TBX1 (n=26). The standard curve with LRP10 as the normalizing gene was used to determine relative gene expression levels. All variables related to gene expression were transformed (log10). BMI: body mass index, FFMI: fat free mas index, FMI: fat mass index, VAT mass: visceral adipose tissue. ***P<0.001. No significant associations were found for body composition and the UCP1 and TBX1 relative gene expression.

GENERAL DISCUSSION

GENERAL DISCUSSION

COLD AS A THERAPEUTIC STIMULUS AGAINST OBESITY. IMPLICATIONS ON ENERGY BALANCE AND BAT RECRUITMENT AND ACTIVATION

The fact that cold exposure (within desirable ranges) is a potential therapeutic stimulus – i.e., it prompts several physiological responses and adaptations which can have protective effects – is not something novel.

Research from birds and mammals' models show that increases in BMR and changes in thermal conductance happen as adaptations to cold environment[1]. Similarly, with decreasing ambient temperatures, and in an effort maintain net heat balance in these animals, CIT response is increased[2]. In fact, even in hypothermia events, where body temperature is decreased beyond the set point, this decrease in temperature seems to have a protective effect, improving survival chances under different insults[3]. In humans, first research on the cold effect over thermoregulation and metabolism dates from decades ago[2,4,5]. By that time, it had already been shown that exposing humans to cold could lead to increases in energy expenditure ranging from approximately 10% to 400% over REE, depending on the exposure conditions. This data pointed some of the potential therapeutic benefits of exposing humans to cold, suggesting that it could be used as a potential strategy to face obesity at long term[6–8] – however, its underlying mechanisms remain to be further ascertained.

In the **Study I,** we showed the physiological responses to mild cold from a comprehensive insight, in order to better understand the underlying mechanisms of non-shivering thermogenesis. In line with previous research[2,9,10], we found that mild cold exposure slightly increases energy expenditure (31.7% above REE), and that it increases fat oxidation (up to 72.6% above resting levels) - specially at the initial moments of cold exposure. We additionally observed that as shivering comes closer: i) skin temperature - mean, proximal and distal - and thermal comfort perception decreases, except the supraclavicular skin temperature, which did not change; ii) superficial muscle increases its electrical activity, clearly contributing to the increase of REE. Taken all together, it seems that non-shivering thermogenesis accounted for a large part of CIT, especially during the initial moments of cold exposure, that is around the first 30 minutes in our cooling protocol. This finding could also provide practical

guidelines for future uses of mild cold as a health promoter stimulus, so that the effect of cold in a short time of period can be maximized while increasing adherence.

Nevertheless, there is controversy regarding the underlying mechanisms of CIT during mild cold exposure. Whether shivering or non-shivering thermogenesis act together or independently, and to what extent each component contributes to CIT still remains unclear[2,6]. In addition, several authors have proposed BAT as one of the main mediators of non-shivering thermogenesis[11,12], whereas others suggest that muscle contribution to is larger. It has been also postulated that BAT and muscle contribute synergistically to non-shivering thermogenesis[13]. As appreciable, there is controversy around this issue. Importantly, a recent study quantified the contribution of deep and centrally located muscles to whole body energy expenditure during cold stress, and compared it to the contribution of BAT, showing that the former was much higher (86 kcal/day vs. 10 kcal/day)[14]. This potentially demonstrates that the role of BAT in human CIT is not important as initially thought. Other tissues such as the heart or liver also contribute to the increase on CIT.

Beyond the specific contribution of each organ/tissue to CIT, and how it is regulated, the small increase that we observed on CIT (~20.83 kcal/h), suggests that the contribution of mild cold to daily energy expenditure might be negligible. This calls into question the feasibility and efficiency of mild cold as a strategy to face obesity at long term[6–8]. Accordingly, previous reports have shown that exposure to an acute cold protocol after 10 to 31 days of cold acclimation (using cold-air or liquid-perfused cooling suits[13,15–17]), increases CIT to a similar extent than the values observed before the cold acclimation; however, these studies failed to find any change in weight or body composition[13,15]. This suggests that the increase in daily energy expenditure induced by CIT may be compensated by adaptive mechanisms (e.g., increase in food intake, decrease in physical activity, increased metabolic efficiency etc.)[18]. Therefore, the implementation of mild cold to harness energy balance and ease a sustainable weight loss remains to be further studied.

It has been also proposed that mild cold exposure may several health benefits (beyond energy balance), many of which seem to be mediated by the recruitment and activation of BAT. For instance, BAT works as a nutrient sink, helping to regulate glucose and lipid metabolism[19–21]. Accordingly, It has been suggested that it mediates

improvements on glucose homeostasis and whole body insulin sensitivity[22]. In addition, those participants with high levels of BAT may be also those who present a decreased cardiovascular risk profile, or viceversa[23–26]. Nevertheless, most of this evidence in humans in inconclusive and ambiguous, for several reasons: i) the variety of methods and protocols used to stimulate, measure, and analyse BAT metabolic activity greatly differ among studies, hampering their comparability; ii) much of this research comes from epidemiological studies – lacking causality -, and consequently there are many potential confounders which could be biasing the relationship between BAT function and certain health outcomes; iii) although BAT is partially mediating these effects (e.g., on glucose and lipid metabolism), most of these changes can be only partially attributable to BAT, and the contribution of other tissues such as skeletal muscle should be considered; and iv) most conclusions on the potential therapeutic effect of mild cold and BAT activation are extrapolated from acute studies - hence it cannot be known whether the same would be observed at long term.

Importantly, most of these studies examining the effects of cold on BAT function, and health outcomes, have been performed under well controlled lab conditions, which greatly differ from those observed in the daily life. However, to know whether the chronic exposure to cold under free-living conditions could have the same effects on BAT recruitment and activity and health outcomes is of great interest. This is important, because people normally spend around 90% of their time indoors, especially in colder regions/seasons[27]. Accordingly, in the **Study II,** we found that those participants exposed to the lowest ambient temperature showed 3-5 times more BAT volume and activity compared to subjects who were exposed to a warmer ambient temperature. A better understanding of the mechanisms underlying the relationship between chronic exposure to cold and BAT function is needed.

Taking together, it seems of great relevance to further explore the potential role of BAT as a strategy to face type 2 diabetes and cardiovascular diseases. The controversy around this issues is further incentivized because short cold acclimation protocols (from 10 days to 4 weeks) have shown great increases on BAT ^{18}F-FDG uptake and its oxidative capacity[13,15,28], although no changes in plasma cardiometabolic markers were observed, except in fasting glucose[28]. In line with this, a recent epidemiological study showed that exposure to cold temperatures is related to

a lower prevalence of dysglicemia and insulin resistance in Spanish adults, but no measurement of BAT activity was performed[29]. In the **Study II,** we also showed that that being exposed to lower temperatures was related to lower fasting glucose concentrations and a lower diastolic blood pressure compared with those participants exposed to warmer temperatures – and this was accompanied by a 3 to 5-fold increase in BAT volume and metabolic activity. Whether cold exposure promotes health benefits at long-term (especially on glucose metabolism), and whether they are independent of BAT metabolic recruitment and activity, needs to be explored. Of note, as most tissues in our body, BAT is a secretory organ[30]. Therefore, many of its potential therapeutic effects and its contribution to the organism regulation, could be performed in a paracrine or endocrine fashion[30].

ROLE OF THE BIOLOGICAL CLOCK ON HUMAN HEALTH: A FOCUS ON ENERGY BALANCE AND BAT ACTIVITY

The biological clock - which dictates how most physiological functions fluctuate and synchronize - is tightly linked to health and disease in humans[31]. This can be observed in the current society, where social habits commonly disrupt the circadian system (chronodisruption), making people more prone to metabolic problems such as obesity, dyslipidemia or impaired glucose tolerance, and the development of certain tumours[32]. Experimental evidence also supports this, showing that circadian disruption can have important implications on cardiometabolic health and other pathologies[31,33,34]. Hence, the correct functioning of the circadian system seems necessary for properly and temporally adjusted behavioural and physiological functions, and *vice versa*.

Nevertheless, we are still far from understanding how the circadian clock and health are linked. For this purpose, we should unravel the entrainment characteristics and mechanisms at all levels of the circadian network, i.e., not only how the SCN synchronizes to the environment, but also to how all cells, tissues, and organs act as a part of an orchestrated, daily programme[31]. Despite its complexity, future studies will aim to map the optimal and sub-optimal entrainment in humans at all levels.

In an attempt to better understand how the circadian system works, several “marker rhythms” have been proposed as a means of assessing its functioning. These marker rhythms are physiological functions driven by the suprachiasmatic nucleus – allowing the timing of internal biological processes to be followed. Marker rhythms

need to be easy to measure in a non-invasive manner, be reliable, have a large amplitude, and have a specific phase relationship with the SCN[35]. In the **Study II,** we used distal skin temperature (DST) – which is also driven by the circadian clock via the autonomous nervous system - as a marker rhythm to examine the relationship of the "circadian status/functionality" and body composition and the cardiometabolic profile in young adults. Interestingly, we found that under free-living conditions, a more flattened, fragmented and less stable DST daily rhythm is associated with a higher BMI, greater body fat accumulation, and a poorer cardiometabolic profile. It is also noteworthy that greater stability and lesser fragmentation of DST daily pattern showed an association with greater cardiorespiratory fitness, a major predictor of cardiovascular health[36]. This seems to be in line with the current knowledge, suggesting that the disruption of the circadian system is associated with obesity and altered metabolism, probably explained by the desynchronization of different organ- or pathway-specific circadian rhythms[33,37]. Indeed, it has been shown that a disrupted DST daily rhythm is a predictor of a lower weight-loss effectiveness[38] and that it is related to certain features of metabolic syndrome[39].

Since BAT may have important clinical implications on some of the above mentioned outcomes – e.g., the cardiometabolic profile – we hypothesized that a worse quality daily pattern of the DST may be also related to a worse BAT function – reflected by a lower BAT volume or metabolic activity. Results from the **Study II** initially showed that a more flattened, fragmented, and less stable DST daily rhythm was associated with a lower BAT ^{18}F-FDG uptake. However, these associations became non-significant after adjusting for sex and the habitual temperature to which subjects were exposed during the week that DST was measured. Taken together, these findings suggest that the relationship between the functioning of the circadian system (indirectly measured by the daily rhythm of DST) and BAT variables is masked by environmental and behavioural factors under free-living conditions, and therefore precludes us from drawing any conclusion. However, this provides important guidelines highlighting the confounding effect of environmental temperature on studies related to any aspect of BAT, including its circadian rhythmicity.

As can be noted, the biological clock is partially determined by behaviour – for instance, exposure to different thermal environments (e.g., cooler temperatures), but

also feeding time and social interactions can modulate it[37,40]. Similarly, the influence of sleep on the biological clock has increased in industrialized societies, since this is often the only moment when we expose our SCN to real darkness[41]. This forms a triangle clock-sleep-behaviour - where the biological clock receives the input from the other 2 components, but at the same time, regulates their fluctuations as part of the whole organism. This triangle is instrumental in balancing health and disease[31]. To examine, therefore, how the wake-sleep cycle, as well as sleep duration and quality are related to different health outcomes is of great interest.

In the **Study IV** we found that sleep duration and quality variables were not related to the cardiometabolic profile of young adults (secondary results), which may look controversial, since an extensive body of epidemiological and experimental evidence has shown that sleep curtailment and disturbances are related to disruption of metabolic and endocrine functions[42–44]. However, this may be explained because our sample was composed of young adults, most of whom had a healthy cardiometabolic profile, and do not present sleep problems typical from other populations (e.g., participants with sleep apnea); this could have masked or weakened the associations. However, we showed that there was a weak inverse relationship between in-bed time and both BMI and VAT mass (after controlling for sex), and suggested that in-bed time may be related to increased risk of obesity through indirect mechanisms. Which are the indirect mechanisms that may explain the relationship between sleep regulation and metabolic homeostasis are not completely understood.

In the **Study IV,** we aimed to examine the role of sleep on BAT metabolic function. We speculated that a shorter sleep duration, worse sleep quality, or/and an altered wake-sleep pattern could be related to BAT metabolic dysfunction. This would be in line with previous reports showing the adverse metabolic effects of sleep-deprivation or sleep quality disruption[42–44]. Nevertheless, we did not find any significant relationship between sleep variables and BAT ^{18}F-FDG uptake and radiodensity in young healthy adults. Indeed, a previous study[45] experimentally proved that participants with type 1 narcolepsy (and therefore largely altered sleep patterns) present a similar sympathetic innervation and metabolic of BAT than healthy controls. This seems controversial, since previous experiments in rodents have shown that BAT function is closely related to sleep regulation[46–52]. In fact, these studies have shown

that, in sleep deprivation protocols, BAT function seems to be increased as a protective mechanism to compensate the large body temperature decrease. This field ensures further experimental research on the relationship between sleep and BAT function in humans.

Thereby, it seems irrefutable that the biological clock and related factors (e.g., behaviour and sleep), are tightly related to health and disease. However, much work is still to be done on mapping the entrainment of physiological functions at all levels, and on understanding the individual influence of each component on health. This will allow us to develop personalised plans, prescribing specific zeitgeber exposures (e.g., timing of light exposure, meals, exercise, or other stimulus such as cold), to maximize the effect of specific interventions on the health outcomes of interest[34].

In the Study III, we aimed to examine whether CIT follows a diurnal/circadian rhythmicity - as previously shown with REE and DIT[53,5455–57]- as well as the metabolic activity of BAT, one of the contributors to CIT. Increasing CIT and BAT metabolic activity have been proposed as potential strategies to counteract positive energy balance and face obesity at long-term. However, it remains unknown whether they follow a diurnal fluctuation, being susceptible of being maximized at specific moments of the day in humans. We found that there were no differences in CIT and cold induced nutrient oxidation rates, neither in their kinetics during a mild cold exposure, across 2 different moments of the day (i.e., morning and evening). Similarly, BAT ^{18}F-FDG uptake was not different across the morning, afternoon and evening. These findings suggest that CIT and BAT ^{18}F-FDG uptake may not exhibit diurnal variations in young healthy adults under these protocols, although these findings should be further corroborated. Even, whether CIT and BAT activity would follow diurnal oscillations under different protocols (unmasking conditions or misalignment protocols), it remains to be seen whether harnessing them at specific times of the day would have implications on daily energy expenditure and body weight regulation at long term. In any case, as science advances, and new fields such as chrono-medicine arise - where the intake of certain drugs at specific moments of the day have larger desired effects and less secondary effects[31] –, it is becoming clearer that individualizing exposure to certain stimuli at specific moments of the day, could convey more health benefits.

EXERCISE AND PHYSICAL ACTIVITY AS STRATEGIES TO RECRUIT AND ACTIVATE BAT AND INDUCE BROWNING

Exercise has been proposed a potential stimulus in the recruitment and activation of brown adipose tissue (BAT)[58], through several mechanisms, such as the canonical sympathetic activity increase, and the release of adrenergic independent factors (e.g., IL-6, meteorin-like, irisin, FGF-21.)[58,59]. However, to date evidence from rodent and human models[10,60–65] is controversial, and seems to indicate that chronic exercise is associated with the downregulation of BAT metabolic activity. Similarly, unpublished data from our research group shows that a 6 month-concurrent exercise programme does not have any effect of BAT ^{18}F-FDG uptake in young adults. The influence of exercise on BAT activity is poorly understood nowadays, and further research is need to better understand the exercise-induced adaptations on

Importantly, to date, no experiments have examined whether an acute bout of exercise induce changes in the physiological and molecular interface of human BAT. Before trying to translate findings from exercise chronic interventions to humans, it is fundamental to determine if acute exercise can modulate BAT function, and if so, through which mechanisms acute exercise is able to modulate it (classical and alternative mechanisms). A step to initially approach this issue may be performed in rodents, and then translated into humans (whenever possible). In the **Study V**, we observed that UCP1 levels tended to decrease after an acute bout of aerobic exercise compared to non-exercise mice. However, no differences between groups were found when we relativized UCP1 to β-tubulin protein concentrations and statistically compared both groups. In addition, we were not able to identify detectable levels of p-p38 MAPK in iBAT, one of the most important molecules on the signalling cascade of canonical UCP1 regulation. Considering that an acute bout of aerobic exercise does not seem to influence UCP1 levels, one might expect that chronic interventions of the same character would neither do, as has been normally observed in rodent experiments[66–77]. Which specific mechanisms could explain the above fact – that exercise seems not to affect rodent BAT, or even induce its "whitening"[66] - remain to be ascertained. One of the possible explanations is that during exercise programmes, skeletal muscle mass increases. Hence, the thermogenic power of the skeletal muscle could partially substitute that of BAT under thermal stress – inducing BAT atrophy[64].

Furthermore, exercise is a highly thermogenic activity, and large energy resources are spent in order to dissipate excess heat and avoid damage to our organism. Hence, it seems coherent that during exercise, BAT thermogenic activity would be the same, or would be even decreased, in order to avoid excessive heat generation[78]. It may be also possible that chronic exercise is not affecting UCP1, but a difference in protein appears because exercise decreases BAT mass[79].

Other alternative mechanisms, such as the secretion of adrenergic independent factors, may help to modulate BAT function[59]. Previous evidence has indicated a potential role for IL-6 mediating the thermogenic activity of BAT, and its effects on metabolism[80–84]. As during acute exercise, systemic IL-6 concentrations are generally increased[85,86], we aimed to examine whether this could be a mechanism linking exercise and BAT function. In the **Study V,** we showed that an acute bout of aerobic exercise is not likely to have any effect on UCP1 protein levels through the IL-6/JAK/STAT3 pathway. Nevertheless, based on our results, we speculated that BAT may be secreting IL-6 to the bloodstream[30] - as previously shown in culture media[81] –, and may be exerting some paracrine or endocrine function, for instance, acting as an energy sensor, or increasing insulin-stimulated glucose uptake in other tissues.

Furthermore, it has been suggested that a phenomenon known as browning - the apparition of brown-like adipocytes within WAT[87] – can be induced by exercise in humans, especially in subcutaneous depots[59,64,88]. In 2012, Boström et al. [89] identified irisin, a PGC-1α dependent and exercise-responsive myokine able to induce browning in WAT depots. Since then, a growing body of evidence has shown that exercise may be more related to the browning of WAT (mainly subcutaneous depots) rather than to the stimulation of classical BAT in rodents [64,66,70,89–91]. In fact, Wu et al.[66] recently showed that exercise antagonistically regulated the thermogenic capacity of classical BAT and subcutaneous WAT in mice, decreasing the former. However, this issue is still controversial[78,92,93]. In humans, evidence has shown little or no effect of chronic exercise on the expression of selected browning genes in the abdominal sWAT[10,78,94,95]. Research is needed to understand these differences between studies observed in mice and humans - although beyond the vast phenotypic differences between species, it has been suggested that results in mice are likely to be influenced by the housing temperatures of their cages[93].

Beyond the effect of exercise, scarce attention has been paid to the role of other modifiable lifestyle behaviours (such as sedentary time and physical activity (PA)) on BAT function, and browning of sWAT. Nowadays, people are spending more time in sedentary behaviours while decreasing PA levels, which has been strongly related to obesity and diabetes. Accordingly, it might be speculated that BAT physiological effects or browning of sWAT could mediate these associations. Thus, in the **Study 6,** we aimed to examine whether sedentary time and PA were associated with BAT ^{18}F-FDG uptake, as well as to the regulation of browning genes in sWAT, in young adults. Until now, only Dinaset al.[96] had shown a positive relationship between subjectively measured PA and BAT activity estimated by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography combined with computed tomography (PET/CT), in subjects with cancer. The same group showed in an independent study[97], that the expression of several browning formation markers was more highly expressed in the sWAT of healthy adult men displaying moderate rather than low PA levels. Nevertheless, these studies presented several design and methodological aspects that may considerably limits the generalization of their results[98]. Contrary to them, we showed that objectively measured PA (at different intensities) was not related to BAT ^{18}F-FDG uptake, neither to the expression of genes related to BAT or browning in sWAT in young healthy adults. Hence, it seems that the relationship between higher levels of PA with decreased obesity and prevalence of several cardiometabolic diseases may not be mediated by BAT function or sWAT browning. It is noteworthy that, UCP1 and TBX1 were scarcely detected in sWAT, indicating that browning in sWAT is unlikely in humans, and that other depots should be further examined[99].

MAIN LIMITATIONS

The results of the present Doctoral Thesis should be interpreted with caution. Specific limitations have been noted for each study throughout the whole document. However, there are some limitations which merit to be highlighted:

i) We used the “shivering threshold” as the end-point of the cooling protocols. The use of a self-reported shivering threshold may be questioned as a valid method to stablish shivering onset, since conscious thermal perception and localization are regulated by the thalamus and cerebral cortex, whereas shivering is controlled by the preoptic area of the hypothalamus[100,101]. In addition, the visual

determination of shivering threshold by researchers is not an objective measurement. Hence, the shivering threshold was only considered an indicator of shivering onset that allowed us to individualize the cooling protocol for every participant. Nevertheless, to the best of our knowledge, no studies have compared yet whether the use of a subjectively determined shivering threshold differs from the onset of shivering determined by EMG. Furthermore, although other methods such as temperature clamping have been proposed[102], the use of shivering threshold has been extensively used and accepted as a valid method to maximize non-shivering thermogenesis and activate BAT[62,103–107].

ii) Despite being the most extended technique to assess BAT, a single static ^{18}F-FDG PET-CT scan has several limitations that might not allow for the accurate estimation of cold-induced BAT metabolic activity[108]. BAT is mainly fuelled by triglycerides obtained by BAT intracellular lipolysis and plasma non esterified fatty acids[11,109–111], whereas we use a glucose analogue (^{18}F-FDG) to estimate its metabolic activity. Whether the present findings will replicate when using other radiotracers such as ^{15}O-oxygen, ^{11}C-acetate or ^{18}F-fluoro-6-thia-heptadecanoic acid (FTHA) to quantify BAT metabolism, remains to be seen. The use of a dynamic PET/CT scan, which allows to assess BAT activity at several temporal moments, should be implemented. In addition, the apparition of new PET/CT scans, with higher resolution and less radiation, will help us to keep advancing on this field.

iii) In the studies 2, 3, and 4, BAT metabolic activity could not be continuously assessed due to the current lack of valid instruments to indirectly assess it (e.g., skin temperature, near infrared resolved spectroscopy, infrared thermography)[112,113]. Similarly, BAT activity could not be assessed at different moments of the day by ^{18}F-FDG-PET/CT, due to ethical problems related with the exposure of participants to high radiation doses. Future studies implementing the continuous assessment of BAT metabolic activity are needed, although this will depend on the development of new and valid techniques to assess it.

iv) In the study 4, we used accelerometry to assess sleep duration and quality. Despite its validity, especially with large cohorts of participants, it does not provide information related to sleep architecture. Future studies should aim to examine the relationship between sleep assessed by polysomnography, and BAT variables. Sleep deprivation interventions should be also performed to further understand whether sleep regulation affects BAT function (causality).

v) In several studies, we examined the diurnal oscillation of different physiological functions under free living and/or masking conditions. Future studies should also aim to carry out these studies under constant routine or misalignment protocols to examine whether results replicate independently of masking and/or behavioural factors (e.g., when examining whether CIT response has diurnal fluctuations).

vi) In the study 5, we use wild-type mice kept in cages at a constant temperature of 23º C. Nevertheless, this temperature is not within the thermoneutral comfort range of mice, causing a cold stress to them which may have affected the results from this study[93].

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CONCLUDING REMARKS

CONCLUDING REMARKS

GENERAL CONCLUSIONS

The results of the present Doctoral Thesis provide a better understanding of the underlying mechanisms of non-shivering thermogenesis, and the potential role of cold exposure during daily life as an efficient stimulus to activate and recruit BAT. We provide evidence that environmental temperature to which participants are exposed should be strongly considered as a masking factor when examining BAT function. Furthermore, we showed that CIT and BAT ^{18}F-FDG uptake do not seem to follow diurnal variations, and that an important component of the biological clock, such as sleep, does not seem to be related to BAT metabolic activity. Finally, we showed that exercise and physical activity may not be adequate strategies to recruit and activate BAT, neither to regulate the expression of genes related to browning in the humans' sWAT, and examine the underlying mechanisms.

SPECIFIC CONCLUSIONS

PART I. Role of cold on energy balance and brown adipose tissue

- **Study I.** Non-shivering thermogenesis seems to importantly contribute to CIT during mild cold exposure, being accompanied by a higher fat oxidative metabolism – especially during the first 30 minutes of cold exposure.

PART II. Role of the biological clock and sleep on energy balance and brown adipose tissue

- **Study II.** A better quality of the DST daily rhythm – used as a proxy of proper of circadian dysfunction - is associated with a lower BMI, greater body fat accumulation, and a better cardiometabolic profile – including greater cardiorespiratory fitness. The potential relationship between the daily rhythm of DST and BAT ^{18}F-FDG uptake is masked by environmental and likely behavioural factors. The subjects exposed to the lowest personal environmental temperature had the lowest DST throughout the day, and had BAT volume and activity values 3-5 times those of subjects exposed to higher temperatures.

- **Study III.** CIT and BAT ^{18}F-FDG uptake, do not seem to have diurnal variations in young healthy adults.
- **Study IV.** Sleep duration and quality appear not to be related to BAT volume or activity (both estimated by ^{18}F-FDG uptake) or BAT radiodensity following cold exposure, in young adults

PART III. Exercise and physical activity as new strategies to recruit and activate brown adipose tissue

- **Study V.** An acute bout of aerobic exercise does not seem to have an effect on UCP1 levels in iBAT through the canonical activation pathway, neither through a new explored one, the IL-6/JAK/STAT3 pathway. However, during acute aerobic exercise, iBAT might release small amounts of IL-6.
- **Study VI.** Objectively measured PA levels are not associated with BAT volume or activity, neither with the regulation of browning markers in the sWAT of young adults.

FUTURE PERSPECTIVES

Despite the great advancement observed during the last years in many of the topics examined and discussed in the present Doctoral Thesis, there many questions that still remain incompletely understood. Future research should aim to:

- Understand which are the underlying mechanisms and clinical implications of cold exposure (especially mild cold) at long term, providing a comprehensive insight; i.e. not only focused on energy balance, but also on parameters related to the cardiometabolic and immunological profile, neural functions, etc. Additionally, studies should examine the feasibility of these interventions to be implemented as therapeutic strategies.

- Investigate which factors influence people to seek chronic exposure to lower temperatures, and thus rely more strongly on thermogenic processes. Then, strategies aiming to exploit these factors, and increase the exposure of people during their daily life to low temperatures could be prompted.

- To understand how the entrainment of different physiological functions are related to health and disease. To map how exposure to zeitgebers at specific times influence our circadian functions (at all body levels), will help us to find sub-optimal and optimal entrainment times. A focus on metabolism and thermoregulation is essential.

- To investigate in depth, the mechanisms by which short or poor sleep could be related to metabolic and endocrine disruption in humans, and how sleep regulation is linked to our thermoregulation system.

- To understand the biological and clinical significance of BAT, and which is its main role in humans.
    - Does BAT importantly contribute to thermoregulation in adult humans?
    - Could BAT actually help humans face obesity?
    - Which is its function beyond heat production (e.g., as an endocrine organ, contribution to crosstalk between organs, etc.,)?
    - Does BAT as a nutrient sink have real implications on metabolic homeostasis o cardiovascular disease?
    - Given its canonical thermogenic function, could BAT modulate immunological processes (such as inflammation or fever)?

- To investigate potential strategies beyond pharmacological approaches – i.e., related to lifestyle behaviours - which may be useful to recruit and activate human BAT. In the case of exercise, there is a need to understand the acute effects of exercise on BAT function, both at physiological and molecular levels. Then, the effect of different types of exercise interventions modifying the main characteristics (type, volume, intensity, etc.), should be examined on BAT function. Similarly, to further investigate how different exercise interventions affect browning of specific adipose tissue depots (beyond the abdominal sWAT) should be explored.

APPENDICES

PAPERS DERIVED FROM THE DOCTORAL THESIS

PUBLISHED & INCLUDED IN THIS DOCTORAL THESIS

1. **Acosta FM,** Martinez-Tellez B, Sánchez-Delgado G, Alcántara JMA, Acost-Manzano P, Morales-Artacho J, Ruiz JR. Physiological responses to acute cold exposure in young lean men. *PLoS One*. May 2018. doi:10.1371/journal.pone.0196543

2. **Acosta FM,** Martinez-Tellez B, Blondin DP, Haman F, Resen PCN, Llamas-Elvira JM, Martínez-Nicolas A, Ruiz JR. Relationship between the Daily Rhythm of Distal Skin Temperature and Brown Adipose Tissue ^{18}F-FDG Uptake in Young Sedentary Adults. *J Biol Rhythms*. 2019;34(5):533-550. doi:10.1177/0748730419865400

3. **Acosta FM,** Sanchez-Delgado G, Martinez-Tellez B, Migueles JH, Amaro-Gahete FJ, Rensen PCN, Llamas-Elvira JM, Blondin DP, Ruiz JR. Sleep duration and quality are not associated with brown adipose tissue volume or activity - as determined by ^{18}F-FDG uptake, in young, sedentary adults. *Sleep*. August 2019. doi:10.1093/sleep/zsz177

4. **Acosta FM,** Martinez-Tellez B, Sanchez-Delgado G, Migueles JH, Contreras-Gómez MA, Martinez-Avila WD, Mercha-Ramirez E, Alcantara JMA, Amaro-Gahete FJ, Llamas-Elvira JM, Ruiz JR. Association of objectively measured physical activity with brown adipose tissue volume and activity in young adults. *J Clin Endocrinol Metab*. August 2018. doi:10.1210/jc.2018-01312

IN PREPARATION & INCLUDED IN THIS DOCTORAL THESIS

1. **Acosta FM,** Sánchez-Delgado G, Martínez-Téllez B, Llamas-Elvira JM, Ruiz JR. Cold induced thermgenesis and BAT ^{18}F-FDG uptake diurnal variations in young sedentary adults. *Under review*

2. **Acosta FM,** Febbrraio, MA. Effect of an acute bout of aerobic exercise on UCP1 and IL-6 levels in brown adipose tissue. *Short report in preparation*

NOT INCLUDED IN THIS DOCTORAL THESIS

***Equally contribution of authors**

1. **Acosta FM*,** Berchem J*, Martinez-Tellez B, Sanchez-Delgado B, Alcantara JMA, Ortiz-Alvarez L, Hamoka T, Ruiz JR. Near-Infrared Spatially Resolved Spectroscopy as an Indirect Technique to

Assess Brown Adipose Tissue in Young Women. *Mol Imaging Biol*. 2019;21(2):328-338. doi:10.1007/s11307-018-1244-5

2. Arias-Téllez MJ*, **Acosta FM*,** García-Rivero Y, Pascual-Gamarra JM, Merchán-Rámirez E, Martínez-Téllez B, Silva AM, Almanza López J, Llamas-Elvira JM, Ruiz JR. Neck adipose tissue accumulation is associated with higher overall and central adiposity, a higher cardiometabolic risk, and a pro-inflammatory profile in young adults. *Under review*

3. Arias-Téllez MJ*, **Acosta FM*,** Hidalgo JM, Pascual-Gamarra JM, Merchán-Rámirez E, de Lucena-Martins CM, Llamas-Elvira JM, Martinez-Tellez B, Ruiz JR. Objectively measured sedentary time and physical activity are associated with neck adipose tissue in young sedentary adults. *Under review.*

4. **Acosta FM,** Sánchez-Delgado G, Martínez-Téllez B, Llamas-Elvira, Ruiz JR. Relationship between immunometabolic markers and BAT ^{18}F-FDG uptake in young sedentary adults. *In preparation*

5. Amaro-Gahete FJ***, Acosta FM*,** Migueles JH, Ponce-González JG, Ruiz JR. Association of sedentary and physical activity time with maximal fat oxidation in sedentary adults. *Accepted in Scandinavian Journal of Medicine & Science in Sports.*

6. **Acosta FM,** Acosta-Manzano P, Amaro-Gahete FJ, Ruiz JR**.** Dose response effect of a concurrent exercise intervention on the inflammatory profile of young adults: a randomized controlled trial. *In preparation*

CURRICULUM VITAE

1. Personal Information

Francisco Miguel Acosta Manzano
Born: August 13th 1992, Granada, Spain
acostaf@ugr.es / acostaf@go.ugr.es

2. Scientific and clinical education

05/2018 – present	Free assistance to undergraduate degree in Medicine, University of Granada
10/2016 – 04/2020	PhD thesis of Biomedical Sciences, University of Granada entitled: "Effect of physical exercise, cold and factors related to circadian rhythmicity on the energy balance in humans"
07/2019 – 10/2019	Research internship of 3 months at MIPS, University of Monash (Melbourne, Australia)
07/2017 – 09/2018:	Research Internship of 2 months at the University of Ottawa (Canada)
07/2017 – 07/2017	Research Internship of 1 week at the University of Leeds (England).
07/2016 – 07/2016	Research Internship of 1 week at LUMC (the Netherlands)
10/2015 – 07/2016:	Master in Research on Physical activity and Health, University of Granada
07/2015 – 09/2015:	Research Internship of 2 months at University of Utretch (the Netherlands)
09/2013 – 06/2014:	Erasmus Student Internship of 8 months at University-AWF, Krakow, Poland
09/2011 – 07/2015:	4-year undergraduate degree in Sport Sciences, University of Granada.
09/2010 – 07/2011:	1-year undergraduate degree in Physical Education, University of Granada.

3. Research experience

02/2019 – present:	-**Project Manager** at Dept. Physical Education and Sport, UGR. **Project:** Effect of capsinoids intake and physical exercise on energy metabolism and brown adipose tissue in adults. ACTIFOX study. **Principal Investigator:** Jonatan R. Ruiz **Funding:** Spanish Ministry €25,000
09/2015 – present:	-**Researcher** at Dept. Physical Education and Sport, UGR. **Project:** Activating brown adipose tissue through exercise in young men and women adults: The ACTIBATE study. **Principal Investigator:** Jonatan R. Ruiz **Funding:** Spanish Ministry €175,000; others: €150,000
01/2015 – 07/2015:	-**Student assistant** at Dept. Physical Education and Sport, UGR. **Project:** Effect of balneotherapy and physical exercise in hot water on the body temperature and pain in women with fibromyalgia. **Principal Investigator:** Victor Segura Jiménez **Funding:** Spanish Ministry €4,500
01/2015 – 07/2015:	-**Project manager** at Dept. Physical and Sports Education, UGR. Hospital de Traumatología (Virgen de las Nieves) **Project**: Influence of physical activity on the severity and incidence of Psoriasis **Supervisors**: Pablo Tercedor y Jesús Tercedor
09/2014 – 07/2015:	-**Student assistant** at Dept. Physical and Sports Education, UGR **Project**: Physical activity in women with fibromyalgia: effects on pain, health and quality of life **Supervision**: Manuel Delgado Fernández

4. Management activities (and others)

11/2018 – 12/2018	Scientific committee of ACTIBATE symposium, Granada, Spain.
04/2019 – present	Reviewer of the International Journal of Obesity (IJO, IF: 5.34).

5. Personal grants

2018: Short internship grant: 3 months to visit the MIPS, at the University of Monash, Australia. Funded by the University of Granada (€3,300).
2018: Conference speaker grant: EDCS Programme, University of Granada (€400).
2016: "Technical support and Management Staff" 3 years, full time contract, University of Granada. Spanish Ministry of Economy, Industry and Competitiveness (€36,000). REF: PTA2016-12264-I.
2015: Short internship grant: 2 months to visit the University of Utretch (€700).
2015: Initiation to research grant: full year (partial time). Plan Propio de Investigación. University of Granada (€1100).

6.Published papers (n=24):

*equally contributed

Acosta FM, Martinez-Tellez B, Sánchez-Delgado G, Alcántara JMA, Acost-Manzano P, Morales-Artacho J, Ruiz JR. Physiological responses to acute cold exposure in young lean men. PLoS One. May 2018. doi:10.1371/journal.pone.0196543. (IF = 2.8).

Acosta FM, Martinez-Tellez B, Blondin DP, Haman F, Resen PCN, Llamas-Elvira JM, Martínez-Nicolas A, Ruiz JR. Relationship between the Daily Rhythm of Distal Skin Temperature and Brown Adipose Tissue ^{18}F-FDG Uptake in Young Sedentary Adults. *J Biol Rhythms*. 2019;34(5):533-550. doi:10.1177/0748730419865400. (IF: 3.9).

Acosta FM, Sanchez-Delgado G, Martinez-Tellez B, Migueles JH, Amaro-Gahete FJ, Rensen PCN, Llamas-Elvira JM, Blondin DP, Ruiz JR. Sleep duration and quality are not associated with brown adipose tissue volume or activity - as determined by ^{18}F-FDG uptake, in young, sedentary adults. *Sleep*. August 2019. doi:10.1093/sleep/zsz177. (IF: 5.14).

Acosta FM*, Berchem J*, Martinez-Tellez B, Sanchez-Delgado B, Alcantara JMA, Ortiz-Alvarez L, Hamoka T, Ruiz JR. Near-Infrared Spatially Resolved Spectroscopy as an Indirect Technique to Assess Brown Adipose Tissue in Young Women. *Mol Imaging Biol*. 2019;21(2):328-338. doi:10.1007/s11307-018-1244-5. (IF = 3.5).

Acosta FM, Martinez-Tellez B, Sanchez-Delgado G, Migueles JH, Contreras-Gómez MA, Martinez-Avila WD, Mercha-Ramirez E, Alcantara JMA, Amaro-Gahete FJ, Llamas-Elvira JM, Ruiz JR. Association of objectively measured physical activity with brown adipose tissue volume and activity in young adults. *J Clin Endocrinol Metab*. August 2018. doi:10.1210/jc.2018-01312. (IF= 5.8).

Amaro-Gahete FJ*, **Acosta FM*,** Migueles JH, Ponce-González JS, Ruiz JR. Association of habitual sedentary and physical activity time with maximal fat oxidation during exercise in young and middle-aged adults. *Scandinavian Journal of Medicine & Science in Sports*. (IF=3.63).

Acosta-Manzano P, **Acosta FM,** Femia P, et al. Association of sedentary time and physical activity levels with immunometabolic markers in early pregnancy: The GESTAFIT project. *Scand J Med Sci Sports*. 2020;30(1):148-158. doi:10.1111/sms.13547. (IF: 3.62).

Acosta-Manzano P, Rodriguez-Ayllon M, **Acosta FM**, Niederseer D, Niebauer J. Beyond general resistance training. Hypertrophy versus muscular endurance training as therapeutic interventions in adults with type 2 diabetes mellitus: A systematic review and meta-analysis.

Obes Rev. February 2020:obr.13007. doi:10.1111/obr.13007. (IF=8.2).

Alcantara, Plaza-Florido, Amaro-Gahete, **et al**. Impact of Using Different Levels of Threshold-Based Artefact Correction on the Quantification of Heart Rate Variability in Three Independent Human Cohorts. *J Clin Med*. 2020;9(2):325. doi:10.3390/jcm9020325. (IF=5.7)

Amaro-Gahete FJ, Sanchez-Delgado G, Alcantara JMA, **et al.** Impact of data analysis methods for maximal fat oxidation estimation during exercise in sedentary adults: Data analysis maximal fat oxidation. *Eur J Sport Sci*. 2019;19(9):1230-1239. doi:10.1080/17461391.2019.1595160

Amaro-Gahete FJ, Sanchez-Delgado G, Alcantara JMA, **et al**. Energy expenditure differences across lying, sitting, and standing positions in young healthy adults. *PLoS One*. 2019;14(6):e0217029. doi:10.1371/journal.pone.0217029

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. *Sci Rep*. 2017;7(1):1-12. doi:10.1038/s41598-017-10444-5. (IF = 4.3).

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*. 2018;0(0). doi:10.1016/j.clnu.2018.05.026. (IF = 4.5).

Martinez-Tellez B, Adelantado-Renau M, Acosta FM, **et al.** The Mediating Role of Brown Fat and Skeletal Muscle Measured by 18F-Fluorodeoxyglucose in the Thermoregulatory System in Young Adults. *Obesity*. 2019;27(6):963-970. doi:10.1002/oby.22461. (IF=4.1).

Martinez-Tellez B, Sanchez-Delgado G, Alcantara JMA, **et al.** Evidence of high 18F-fluorodeoxyglucose uptake in the subcutaneous adipose tissue of the dorsocervical area in young adults. *Exp Physiol*. 2019;104(2):168-173. doi:10.1113/EP08742. (IF=2.7).

Martinez-Tellez B, Xu H, Sanchez-Delgado G, **et al**. Association of wrist and ambient temperature with cold-induced brown adipose tissue and skeletal muscle [^{18} F]FDG uptake in young adults. *Am J Physiol Integr Comp Physiol*. 2018;315(6):R1281-R1288. doi:10.1152/ajpregu.00238.2018. (IF= 3.1).

Martinez-Tellez B, Garcia-Rivero Y, Sanchez-Delgado G, **et al.** Supraclavicular skin temperature measured by iButtons and 18F-fluorodeoxyglucose uptake by brown adipose tissue in adults. *J Therm Biol*. 2019;82:178-185. doi:10.1016/j.jtherbio.2019.04.006. (IF=2.1).

Martinez-Tellez B, Perez-Bey A, Sanchez-Delgado G, **et al.** Concurrent validity of supraclavicular skin temperature measured with iButtons and infrared thermography as a surrogate marker of brown adipose tissue. *J Therm Biol*. 2019;82:186-196. doi:10.1016/j.jtherbio.2019.04.009. (IF=2.1).

Migueles JH, Cadenas-Sanchez C, Rowlands A V., **et al.** Comparability of accelerometer signal aggregation metrics across placements and dominant wrist cut points for the assessment of physical activity in adults. *Sci Rep*. 2019;9(1):1-12. doi:10.1038/s41598-019-54267-y. (IF=4).

Sanchez-Delgado G, Martinez-Tellez B, Garcia-Rivero Y, **et al.** Association between brown adipose tissue and bone mineral density in humans. *Int J Obes*. December 2018:1. doi:10.1038/s41366-018-0261-4. (IF=5.2).

Sanchez-Delgado G, Alcantara JMA, **Acosta FM,** et al. Estimation of non-shivering thermogenesis and cold-induced nutrient oxidation rates: Impact of method for data selection and analysis. *Clin Nutr*. 2019;38(5):2168-2174. doi:10.1016/j.clnu.2018.09.009. (IF = 4.5).

Sanchez-Delgado G, Martinez-Tellez B, Garcia-Rivero Y, **et al.** Brown Adipose Tissue and Skeletal Muscle 18F-FDG Activity After a Personalized Cold Exposure Is Not Associated With Cold-Induced Thermogenesis and Nutrient Oxidation Rates in Young Healthy Adults. *Front Physiol*. 2018;9. doi:10.3389/fphys.2018.01577. (IF=3.4).

Sanchez-Delgado G, Martinez-Tellez B, Olza J**, et al.** Activating brown adipose tissue through exercise (ACTIBATE) in young adults: Rationale, design and methodology. *Contemp Clin Trials*. 2015;45:416-425. doi:10.1016/j.cct.2015.11.004. (IF=2.1).

Sanchez-Delgado G, **Acosta FM**, Martinez-Tellez B, et al. Brown adipose tissue volume and 18F-fluorodeoxyglucose uptake are not associated with energy intake in young human adults. *Am J Clin Nutr*. December 2019. doi:10.1093/ajcn/nqz300. (IF=6.6).

Papers under review (n=2)

*equally contributed

Arias Téllez MJ*, **Acosta FM*,** García-Rivero Yolanda, Pascual-Gamarra JM, Merchán-Ramírez E, Martínez-Téllez B, Silva MA, Almanza López J, Llamas-Elvira JM, Ruiz JR. Neck adipose tissue accumulation is associated with higher overall and central adiposity, a higher cardiometabolic risk, and a pro-inflammatory profile in young adults.

Arias Téllez MJ*, **Acosta FM*,** Pascual-Gamarra JM, García-Rivero Yolanda, Merchán-Ramírez E, Martínez-Téllez B, Silva MA, Almanza López J, Llamas-Elvira JM, Ruiz JR. Objectively measured sedentary time and physical activity are associated with neck adipose tissue.

7. Conferences attended
Invited upon abstract submission

2019/06	CPH BAT, Copenhagen. "Diurnal rhythm of brown adipose tissue 18F-FDG uptake and cold induced thermogenesis in young adults".
2019/05	Exercise is Metabolism, Cell Press, Sitges, Barcelona. "Objectively measured physical activity levels are not associated with brown adipose tissue and skeletal muscles glucose uptake in young adults"
2018/11	International Symposium: Role of Brown Adipose Tissue in Human Health. "Are sleep time and quality related to brown adipose tissue function in humans?".
2018/10	VI Congress: EXERNET Simposium. Research in exercise, health and wellbeing: "Exercise is medicine". Pamplona. **Oral presentation:** "Physical activity levels do not predict brown adipose tissue and skeletal muscle glucose uptake in humans".
2018/10	VII International Conference on the Physiology and Pharmacology of Temperature Regulation (PPTR), Split, Croatia. **Oral presentation:** "Wrist skin temperature daily rhythm predicts brown adipose tissue 18F-FDG uptake in young adults".
2017/06	III International congress of PhD students of University of Granada (JIFFI) congress, Granada, Spain. **Oral presentation:** "Asociación entre el nivel de actividad física y la captación de glucosa del tejido adiposo marrón y músculo esquelético en adultos jóvenes.

2017/06	International Simposium: active brains for all: Exercise, cognition and mental health. "Objectively measured physical activity and mental health in sedentary young adults".
2017/05	II International congress of PhD students of University of Granada (JIFFI) congress, Granada, Spain. **Oral presentation**: "Effect of acute cold exposure on energy expenditure in young adults"
2016/10	V Congress: EXERNET Simposium. Research in exercise, health and wellbeing: "Exercise is medicine". "Effect of acute mild cold exposure on adaptive thermogenesis in young adults".

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The human body is a complex and living engine, which is the result of the constant interaction of the cells and components which form our organism.

This engine, as the one of any machine, is composed by a set of gears that need to be perfectly greased and synchronized. If any of the components which form the whole, fail, the whole system will break.

In other words, the correct working of our organism depends on the correct interaction of its components at all levels (cell, tissues, organs, systems), and a perfect timing/syncrhonization of them is necessary for a proper working of physiological functions.

The desynchronization or misalignment of these functions will derive in homeostasis disruption and disease.