PROGRAMA DE DOCTORADO EN BIOMEDICINA

ROLE OF BROWN ADIPOSE TISSUE IN THE THERMOREGULATORY SYSTEM AND PHYSICAL FITNESS: THE ACTIBATE STUDY

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UNIVERSIDAD DE GRANADA

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The ACTIBATE study

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Editor: Universidad de Granada. Tesis doctorales
Cover design & Layout: Manuel Padilla Jódar

International Doctoral Thesis / Tesis Doctoral Internacional

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2018

Editor: Universidad de Granada. Tesis Doctorales
Autor: Borja Manuel Martínez Téllez
ISBN: 978-84-1306-052-1
URI: http://hdl.handle.net/10481/54476

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Rol del tejido adiposo pardo en el sistema termorregulador y la condición física: Estudio ACTIBATE

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A mi padre, madre y hermano.

To my father, mother and brother.

“La actividad más importante que un ser humano puede lograr es aprender para entender, porque entender es ser libre.”

“The highest activity a human being can attain is learning for understanding, because to understand is to be free”

Baruch Spinoza
(1632-1677) Amsterdam

Research projects and funding

The present Doctoral Thesis was performed as a result of the cross-sectional study of the ACTIBATE study (http://profith.ugr.es/actibate?lang=en), which was was supported by:

- Spanish Ministry of Economy and Competitiveness (PTA 12264), Fondo de Investigación Sanitaria del Instituto de Salud Carlos III (PI13/01393).
- Retos de la Sociedad (DEP2016-79512-R), Fondos Estructurales de la Unión Europea (FEDER).
- Spanish Ministry of Education (FPU 13/04365, 14/04172, 15/04059, 16/05159, 16/028028 and 17/01523).
- Fundación Iberoamericana de Nutrición (FINUT).
- Redes temáticas de investigación cooperativa RETIC (Red SAMID RD16/0022).
- AstraZeneca HealthCare Foundation.
- Vegenat S.A.
- University of Granada, Plan Propio de Investigación 2016, Excellence actions: Units of Excellence; Unit of Excellence on Exercise and Health (UCEES).

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ABSTRACT

Since it is known that humans adults present metabolic active brown adipose tissue (BAT), numerous studies have focused in elucidate the role of this tissue in the metabolism as possible therapy to combat obesity or type 2 diabetes. However, its role in the thermoregulatory system or its relation with exercise has not been deeply addressed. In addition, the current techniques to evaluate the physiological responses of this tissue present several limitations. Therefore, the main aims of the present International Doctoral Thesis were: (i) to provide new tools to improve human skin temperature and BAT quantification; (ii) to study the role of BAT in the human thermoregulatory system and in the metabolic profile of young adults, and (iii) to study its relationship with physical fitness.

We have developed a software for improving the measurement of skin temperature (**chapter 2**) and detected differences in the outcomes of skin temperature quantified by different equations (**chapter 3**). Moreover, we confirm that iButtons are measuring by both sides and we propose a feasible solution (**chapter 4**). We have designed a cooling protocol that was able to activate BAT in young adults (**Chapter 5**), and observe that BAT volume and activity highly depends on the threshold selected (**chapter 6**). We show that the standardized uptake value threshold determines BAT outcomes more than the Hounsfield units threshold. We have also shown that the most used threshold in literature is missing around de 41.4% of the total BAT metabolic activity (**chapter 7**). In addition, we show that supraclavicular skin temperature is not related with BAT activity, and that infrared thermography or iButtons could not be valid instrument to assess BAT activity (**chapter 8**). We describe that wrist skin temperature or the personal level of environmental temperature are related with BAT and skeletal muscles glucose uptake (**chapter 9**), and show that BAT mediates the relationship between the personal level of environmental temperature and wrist skin temperature (**chapter 10**). We have also shown that skin temperature responses to a meal-induced intake is different in women than in men (**chapter 11**). We observe that young adults have higher glucose uptake in the dorsocervical area in comparison with other areas and we suggest that it could be interscapular BAT (**chapter 12**). We show that handgrip strength is positively and significantly related with BAT (**chapter 13**). Lastly, we illustrate that metabolically healthy overweight-obese individuals had higher levels of BAT volume and activity and better thermoregulatory system in comparison to metabolically unhealthy counterparts (**chapter 14**).

The results of the present thesis enhance our understanding about how to quantify human skin temperature and BAT, and the possible role of BAT in the thermoregulatory system and in the metabolic profile of young adults, as well as its relation with physical fitness.

RESUMEN

Tras el descubrimiento de que el tejido adiposo pardo (TAP) es metabólicamente activo en humanos adultos, el número de estudios centrados en estudiar el rol del TAP en el metabolismo energético han crecido exponencialmente. Sin embargo, en humanos se desconoce cuál es la función de dicho tejido en el sistema termorregulador o su relación con el ejercicio; una de las razones puede ser que las técnicas actuales para cuantificar el TAP y sus respuestas fisiológicas presentan limitaciones. Por lo tanto, los objetivos de la presente Tesis Doctoral Internacional fueron: (i) proporcionar nuevas herramientas para mejorar las mediciones de la temperatura de la piel y la cuantificación del TAP; (ii) estudiar el rol del TAP en el sistema termorregulador y en el perfil metabólico de adultos jóvenes, (iii) además de estudiar su relación con la condición física. Para ello, hemos desarrollado un nuevo software que mejora el análisis de la temperatura de la piel (**capítulo 2**) y detectamos que los resultados de la temperatura de la piel dependen de la ecuación seleccionada (**capítulo 3**). Además, hemos confirmado que los iButtons miden

por ambos lados y proponemos una solución (**capítulo 4**). A la misma vez, hemos diseñado un protocolo de frío que es capaz de activar el TAP (**capítulo 5**) y hemos observado que la cantidad y actividad del TAP dependen del umbral seleccionado (**capítulo 6**). También mostramos que el umbral de captación de glucosa estandarizada es el que más influye en la cuantificación del TAP, y que el umbral más usado en literatura está perdiendo alrededor del 41.4% de la actividad total del TAP (**capítulo 7**). En el **capítulo 8** se muestra que la temperatura de la piel de la zona supraclavicular no está relacionada con la actividad del TAP. También hemos descrito que la temperatura de la muñeca o la temperatura del ambiente individual de cada individuo está relacionada con la captación de glucosa del TAP y del músculo (**capítulo 9**), al igual que hemos demostrado que el TAP está mediando la relación de la temperatura del ambiente con la temperatura de la muñeca (**capítulo 10**). En otro estudio, observamos que las respuestas de la temperatura de la piel a la ingesta de una comida son más altas en mujeres que en hombres (**capítulo 11**). También observamos que la captación de glucosa en la zona dorsocervical es más alta, sugiriendo que puede ser TAP interescapular (**capítulo 12**). En el **capítulo 13**, mostramos que la fuerza de prensión manual se asocia significativamente y positivamente con el TAP y en el **capítulo 14** que los individuos con sobrepeso-obesidad que son metabólicamente sanos tienen una distribución del TAP diferente en comparación con los que no son sanos.

Los resultados de la presente tesis mejoran nuestro conocimiento sobre cómo debemos cuantificar la temperatura de la piel y el TAP en humanos. Además se muestra el posible rol del TAP en el sistema termorregulador, como en el perfil metabólico de adultos jóvenes. Al igual que se muestra la relación de este tejido con la condición física.

General Introduction

Role of brown adipose tissue in humans

CHAPTER 1

BROWN ADIPOSE TISSUE: DEFINITION AND PHYSIOLOGICAL BASES

In mammals, adipose tissue is mainly found in two forms: white adipose tissue (WAT) and brown adipose tissue (BAT). These two tissues have opposite roles in whole-body energy metabolism. WAT has the ability to store energy contained in glucose and fatty acids in the form of triacylglycerols and to release energy in the form of free fatty acids, whereas BAT has the ability to dissipate energy in the form of heat by oxidation of fatty acids and of glucose[1]. Cold exposure causes the sympathetic nervous system (SNS) to release norepinephrine, which activates brown adipocytes to induce uncoupling protein 1 (UCP1) activity and increase uncoupled aerobic respiration[1]. BAT is characterised by a light pink to dark red tone due to the high vascularization and a cytoplasm containing small lipid droplets and a large amount of mitochondria[1]. This contrasts with white adipocytes, whose cytoplasm contains a big single lipid droplet and few mitochondria. High vascularisation is necessary for nutrient and oxygen supply and for heat dissipation. The stored triacylglycerol depots are necessary for fast energy supply, and the SNS innervation ensures the fast activation of these specific adipocytes[2]. For prolonged thermogenesis activity, the tissue receives substrates (i.e. fatty acids and glucose) from the blood. Heat production takes place through the uncoupling of the respiratory chain and adenosine triphosphate (ATP) synthesis, and it is mediated by the UCP1, a unique inner-membrane mitochondrial protein within brown adipocytes[1]. However, it has been shown that UCP1-independent mechanisms could also play a role[3]. Another type of adipocytes called brite (brown-in-white) or beige cells have been found within WAT of both rodents and humans[3]. Brite cells have a multilocular morphology, are enriched in mitochondria, and also express UCP1. They are peculiar in that they share characteristics with white and brown adipocytes. The development of these thermogenically competent cells in WAT (a process called 'browning')[4] is greatly enhanced in response to chronic cold exposure, prolonged β-adrenergic stimulation or exercise, and the occurrence of these cells is associated with resistance to obesity, type 2 diabetes, and other metabolic diseases[4]. Therefore, there is an urgent need to investigate the potential factors able to stimulate browning of white fat without having side effects[4].

BROWN ADIPOSE TISSUE: HISTORICAL PERSPECTIVE

BAT in humans has been acknowledged since the 16th century when a Swiss naturalist Konrad Gessner stated that it was "neither fat, nor flesh [nec pinguitudo, nec caro], but something in between"[5]. Since then, its metabolic significance for human physiology has remained uncertain for centuries. It was for long believed that human BAT was only present in new-borns in order to support non-shivering thermogenesis. It was thought that the number of brown adipocytes decrease after the first perinatal year, and that adult humans had little or no such tissue left. In 1972, Heaton[6] performed dissections of corpses and confirmed the presence of BAT in young adults but not in older people, yet its metabolic activity could not be determined. Few years later, in 1977, Hassi[7] reported that the prevalence of BAT in human adults from northern Finland was about 10%. In 1981, Huttunen et al.[8] observed the presence of BAT in workers exposed to cold ambient temperatures. In the 1990s, radiologists using the radiotracer 18F-fluorodeoxyglucose (18F-FDG) in positron-emission tomography and computed tomography (PET/CT) scan to detect metabolically active tumours[9,10] found areas with high rates of glucose uptake that were symmetrical in nature[9].

These areas were most commonly localised in the supraclavicular and neck regions[11,12]. In 2002, with the use of 18F-FDG-PET/CT scans, Hany et al. [9]suggested that the observed glucose uptake in the supraclavicular and neck regions was actually

mediated by BAT, as radiodensity in these areas indicated negative values, compatible with adipose tissue rather than lean tissues. At the same time, Cohade et al. [13] introduced the term "uptake in the supraclavicular area fat" ("USA-fat"), referring to an abnormal glucose uptake in the supraclavicular area. They also showed that USA-fat was typically bilateral and symmetric, intense, more often multifocal than linear, and located in fat[13]. The presence of active BAT in adult humans and its metabolic significance for human physiology was stated in 2007[11]. In 2009, five independent research groups showed that BAT is present and active in human adults, as judged from the profound uptake of the accumulating glucose analogue 18F-FDG by PET/CT analysis after cold exposure [12,14–17]. Biopsies from regions with a high uptake of 18F-FDG revealed the presence of UCP1[17], providing evidence that the tissue truly was BAT. Since 2009, the number of human BAT-related studies published has increased exponentially (Figure 1).

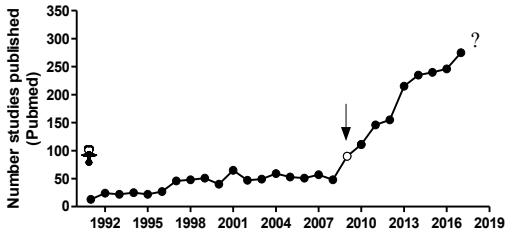

Figure 1. Number of studies published from 1991 to October of 2018 regarding the topic of "brown adipose tissue" or "brown fat". The pacifier symbol represents when the PhD candidate was born, whereas the open circle and the arrow represents the five papers published in 2009 showing that humans have metabolically active BAT.

In the last 9 years we have learnt several things about human BAT and its impact in metabolism:

- The most used technique to quantify human BAT (18F-FDG-PET/CT scan) has several limitations which hampers to perform strong conclusions[18].
- The 18F-FDG uptake by BAT is strongly affected by the outdoor temperature[19]; hence, new and alternative solutions are needed.
- The amount of BAT in the human body represents less than the 1% of the total tissues, in comparison to skeletal muscle, which represents at least the 30% of the total tissue[20]. Thus, the impact of BAT in human metabolism might be less than initially expected.
- BAT has the possibility to act as endocrine tissue, releasing a set of lipokines (batokines), which could impact the human metabolism [21].
- Activation of human BAT by capsinoids, catechins, ephedrine and other dietary components seem promising in humans, but the current evidence have several limitations in the design, which precludes to perform definitive conclusions[22].
- At least in mice, BAT is strongly related in the thermoregulatory responses[23,24]; however, which is the contribution of BAT in the human thermoregulatory system remains unknown.
- Little evidence showed that exercise could downregulate BAT in humans[25] but the physiological role and mechanisms remain undiscovered.

Therefore, further and better design studies are needed in order to elucidate whether BAT has a role in the human metabolism.

BROWN ADIPOSE TISSUE: ROLE IN THE THERMOREGULATORY SYSTEM

The main function of the thermoregulatory system is to keep constant the core body temperature[23]. To do that mammals have two type of mechanisms: behavioural and physiological. Skin blood flow, water evaporation, BAT and skeletal muscles constitute the physiological mechanisms[23]. Skin has a set of cutaneous thermoreceptors based on transient receptor potential (TRP) family of cation.

These cutaneous thermoreceptors are ready to respond to cold and/or hot temperatures. In brief, once that the skin is exposed to cold or hot environment the cutaneous thermoreceptors perceive the ambient temperature and send the information to the preoptic area (POA) in the hypothalamus, which triggers the physiological responses[26]. In a cold environment, the physiological responses are to perform a vasoconstriction and generate heat via shivering (via skeletal muscle) and non-shivering thermogenesis (via skeletal muscle and BAT). However, upon a hot exposure, the physiological responses would be the opposite, the vessels should perform a vasodilatation and the body should start to sweat in order to dissipate heat. The thermal perceptions also play an important role in the thermoregulatory responses. In humans, it is well known that women and men perceived the temperature differently[27]; therefore, their physiological responses are expected to be distinct, yet little is known and especially how BAT could be modulating these responses.

Hardi & Du Bois studied how skin temperature responds to a cold and heat exposure in 1937[28]. After that time, parameters of skin temperature and thermal responses were studied as thermal-physiological responses to different stimulus such as exercise[29], anaesthesia-treatment[30], circadian-rythms[31], or heat[32] and cold[33] environment. Since the rediscovery of BAT and its implication in the thermoregulatory system, interest on skin temperature responses to different stimuli has increased.

Skin temperature assessment

One of the most used methods to measure skin temperature are iButtons, which are small and cheap thermometers that can be attached to the skin. We identified that the current software (One-Wire Viewer) that analyse the data obtained by these devices presents several problems, which hampered the process of data. We also detected that there were different equations as well as different anatomical positions for measuring skin temperature; therefore, we did not know which equation or set of iButtons we should stablished during the data collection of our study, since there is no gold standard for measuring skin temperature. While conducting several experiments in our lab, we realized that iButtons were measuring by both sides, as others also showed[34]. We also detected that supraclavicular skin temperature measured by infrared thermography (IRT) or iButtons, have been postulated as indirect marker of BAT activity[35–37], but we did not know its validity for estimating human BAT.

BAT methodology

Since the main activator of BAT is cold exposure, in humans is needed to cold down the participants before BAT assessment with the 18F-FDG-PET/CT scan. Studies have used either personalized or fixed cooling protocols for BAT activation[38,39]. The main aim of the personalized cooling protocol is to slightly cool down the water temperature until shivering seems to be detectable[40], although this methodology has several limitations[41] is one of the most used in the field[42]. The human studies using the personalized cooling protocol have performed the shivering threshold test first and then have exposed the participants to two hours of an individualized temperature before BAT quantification in the same day.

Due to logistic reasons, we were not able to replicate the design of these experiments. Furthermore, we detected that almost each study used a different range of standardized uptake value (SUV) or Hounsfield Units (HU) thresholds for quantifying BAT measured by statics 18F-FDG-PET/CT scan. The impact of using different threshold on the quantification of human BAT is unknown. We also questioned whether the subcutaneous adipose tissue (SAT) of different parts of human body had the same 18F-FDG uptake.

Thermoregulatory responses

Outdoor ambient temperature strongly affects human BAT[43]. Previous studies have used data from the National Agency of

Meteorology, however most of the participants spent their time indoors during cold and hot months[44]. Therefore, the outcomes obtained by these Meteorology Agencies could not be a good tool to measure the outdoor exposure of the participants. Furthermore, since humans are exposed to cold/hot environment, this exposure has an impact on their BAT volume and activity but how much is affected remains unknown.

Humans, eat several times a day and it is known that this have an impact on the human metabolism[45]. Furthermore, the National Institutes of Health postulated that men and women should be studied independently since they are biologically different[46]. Therefore, whether the thermic effect of food (TEF) assessed by measuring skin temperature after a liquid meal is different in men and women has not been studied.

BROWN ADIPOSE TISSUE AND EXERCISE: PHYSICAL FITNESS

Exercise increases energy expenditure and heat production. Since BAT produces heat and consumes energy, it would be expected that BAT is downregulated in response to exercise[47]. However, exercise stimulates several metabolic pathways that upregulate BAT and/or browning [4,48,49]. Besides secretion of potentially BAT-activating catecholamines[50], exercise increases BAT activators that act independently of stimulation of the SNS, including peptides/hormones. These factors can act on BAT function in an endocrine, paracrine, or even autocrine manner. To date, most empirical evidence regarding the effect of exercise on BAT is derived from animal models, and results are controversial[51]. Some studies have shown a decrease in BAT UCP1 expression[52], while others reported an increased expression of BAT UCP1. There is no consensus about the physiological meaning of physical activity (PA)/exercise-induced browning, and several theories have been proposed[53]. Moreover, it has been suggested that moderate intensity exercise mimics shivering, and therefore induces a similar secretome as in shivering, which would contribute to BAT recruitment and activation, thereby increasing non-shivering thermogenesis capacity[54]. Finally, others have argued that the browning effect represents a compensatory response to the loss of insulation produced by exercise-induced loss of fat mass[55].

In humans, few preliminary studies have been conducted examining the effect of exercise on BAT metabolism. In a cross-sectional study with cancer patients, Dinas et al. [56] showed that levels of thermoneutral 18F-FDG uptake by BAT were positively associated with subjectively measured PA. However, this study presents several methodological limitations that could have biased the findings [57]. In another study, the same group [58] showed that the participants who reported moderate PA levels had higher expression of browning markers in abdominal scWAT compared to the participants who reported low levels of PA, whereas levels of UCP1 expression were similar. Recently, we found that BAT was not related with PA measured objectively in a cohort of young healthy humans[59]. In 2015, Vosselman et al.[60] showed that endurance-trained men had lower levels of 18F-FDG uptake by BAT compared to sedentary controls, whereas no differences in abdominal scWAT browning markers expression between cohorts were observed. Similarly, Singhal et al.[61] reported that endurance-trained women presented lower levels of 18F-FDG uptake by BAT compared to untrained-women. Another observational study (n=2 endurance athletes and n=10 untrained individuals) also reported the same finding [62]. In summary, preliminary human studies present contradictory findings, possibly related to methodological limitations (e.g. observational designs, PET/CT without cold induction, absence of control group, no control of seasonal effect, exercise type, or fitness level of participants)[63,64]. Properly designed studies (randomised controlled trials) with sufficient sample sizes are urgently needed to elucidate the effect of exercise on human

BAT metabolism. Future studies should also consider that different adaptations can be obtained from different training modalities (i.e. endurance vs. strength training).

ROLE OF BROWN ADIPOSE TISSUE ON THE CARDIOVASCULAR PROFILE

Obesity is considered a pandemia that has increased exponentially during the last decades. A study conducted in 19.2 million participants from 200 countries indicates that by 2025, global obesity prevalence will reach 18% in men and surpass 21% in women[65]. Cardiovascular disease (CVD) is the number 1 cause of death globally. Data from the World Health Organization indicate that an estimated 17.7 million people died from CVD in 2015, representing 31% of all global deaths. In Europe, CVD accounted for 45% of all deaths in 2017, and it was the main cause of death in both men and women in the majority of European countries. People with CVD or at high cardiovascular risk due to the presence of one or more risk factors, such as diabetes mellitus, or dyslipidaemia, need early detection and management. Furthermore, there are a subgroup of obese individuals who possess a healthy metabolic profile, the so called metabolic healthy but obesity (MHO). MHO are obese but do not have dyslipidemia, hyperglycemia, hypertension or type 2 diabetes[66,67]. The opposite condition among obese individuals is most commonly named as metabolically unhealthy obesity (MUO). A recent meta-analysis show that around 35% of individuals with obesity is MHO[68]. In addition, a recent review and meta-analysis has shown that the phenotype of MHO seems to have higher levels of PA and spend less time in sedentary behaviour in comparison to their counterparts[69]. They showed that the levels of cardiorespiratory fitness were higher in MHO vs. MUO, whereas there were no differences regarding muscular strength. Most of the studies included were in older adults (>45 years old)[69], but whether the same findings apply to younger adults is also controversial. Nevertheless, BAT has been postulated as a possible tool in the prevention and treatment of CVD by several reasons[25].

This beneficial effect could be attributed to several mechanisms. First, activated BAT may serve as a metabolic 'sink' that internalises glucose thereby correcting hyperglycaemia. This could be mediated by either the insulin-dependent glucose transporter type 4 (GLUT4) channel or the insulin-independent, norepinephrine-induced GLUT 1 channel 2. Alternatively, activated BAT may secrete adipokines that enhance peripheral insulin sensitivity, thereby improving whole-body glucose metabolism[21]. This hypothesis is supported by two independent studies using BAT transplantations[70,71] in which glucose homeostasis was improved regardless of insulin[71] and coincided with increased plasma levels of leptin, adiponectin[71]. Hence, to study whether MHO vs. MUO phenotype present different levels of BAT or thermoregulatory responses remains unexplored.

The present International Doctoral Thesis is structured in four parts. Part 1 includes a set of methodological studies focused on the skin temperature assessment. Part 2 includes methodological studies focused on the improvement of BAT quantification in humans. Part 3 focuses on the relation of BAT in the thermoregulatory system. Part 4 studies the possible relationship of physical fitness and BAT and applies all acquire knowledge to the phenotypes of metabolically healthy but overweight-obese (MHOO) and metabolically unhealthy but overweight-obese (MUOO) young adults.

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Aims

The main aims of this International Doctoral Thesis were: (i) to provide new tools to improve human skin temperature and BAT quantification (Part 1 and 2); (ii) to study the role of BAT in the human thermoregulatory system (Part 3); (iii) To study the relation between physical fitness and BAT, as well as to elucidate the role of BAT and the thermoregulatory responses in a cohort of MHOO and MUOO young adults (Part 4).

The aims mentioned above are approached by 13 objectives:

Part 1: The importance of understanding the instruments: skin temperature assessment

Chapter 2: To develop a software with the main aim of making the task of programming, downloading, and analysing the massive amount of data generated by iButtons effortless, intuitive, time-efficient, and user-friendly.
Chapter 3: To study whether the most used equations to estimate parameters of skin temperature in human BAT studies measure the same in young lean men.
Chapter 4: To determine whether iButtons measure at both sides, and to validate a solution for improving the temperature measurement with iButtons.

Part 2: The importance of understanding the instruments: brown adipose tissue methodology

Chapter 5: To determine the effect of a novel personalized cooling protocol where the shivering threshold was measured on a separate day, on BAT volume and activity in young adults.
Chapter 6: To compare and quantify BAT volume and activity following BARCIST 1.0 recommendations against the most commonly used HU and standardized uptake values SUV thresholds in three different cohorts of men including young lean adults, young overweight/obese adults, and middle-aged overweight/obese adults.
Chapter 7: (i) To examine the influence of SUV and HU thresholds on the quantification of BAT.

Part 3: Brown adipose tissue and thermoregulation

Chapter 8: (i) To study the concurrent validity of skin measured by iButtons and infrared thermography. (ii) To study the association between supraclavicular skin temperature measured by iButtons and infrared thermography with BAT volume and activity quantified by 18F-FDG-PET/CT scan following the current recommendations.
Chapter 9: To study the association of wrist skin temperature and the personal level of environmental temperature with cold-induced 18F-FDG uptake by BAT and skeletal muscle in young adults. We also studied whether the time spent in a certain range of temperature is associated with the 18F-FDG uptake by BAT and skeletal muscles.
Chapter 10: To study the mediating role of BAT and skeletal muscle activity (18F-FDG uptake) between personal level of environmental temperature and skin temperature in young healthy adults. In order to understand the physiological mechanisms, we examined whether the association of the number of hours exposed to a certain personal level of environmental temperature with BAT and skeletal muscle 18F-FDG uptake is mediated by wrist skin temperature as a surrogate marker of skin blood flow mechanisms.
Chapter 11: To determine whether the thermic effect of food with a standardized and individualized liquid meal on skin temperature is different in young men than in women.

Part 4: Clinical Implication

Chapter 12: To study whether the 18F-FDG uptake in the subcutaneous adipose tissue of the dorsocervical area is higher in comparison to the subcutaneous adipose tissue of the other area, and (ii) to study whether the glucose uptake by subcutaneous adipose tissue of these zones correlate with the glucose uptake of supraclavicular BAT, in young adults.

Chapter 13: To determine the association of physical fitness (cardiorespiratory and muscular fitness) with 18F-FDG uptake by BAT and skeletal muscle upon a cold exposure in young adults.

Chapter 14: To determine differences in the distribution of BAT and in the thermoregulatory responses, levels of physical activity and fitness in a cohort of metabolically healthy overweight-obese vs. metabolically unhealthy overweight-obese young adults.

Material & Methods, Results and Discussion

Table 1. Summary of the characteristics of the chapters included in the present International Doctoral Thesis.

Chapter	Design	Participants	Variables studied
Part 1: The importance of understanding the instruments: skin temperature assessment			
2. Temperatus ® software: a new tool to efficiently manage the massive information generated by iButtons.	Description of the Temperatus ® software.	N.A.	N.A.
3. Differences between the most used equations in brown adipose tissue human studies to estimate parameters of skin temperature in young lean men.	Cross-sectional. Methodological study.	11 men; age: 23.4±0.5 years; BMI: 23.2±0.4kg/m2.	Skin temperature (iButton).
4. A methodological approach to improve skin temperature measurement using iButtons in human cold-induced studies.	Experimental. Methodological study.	1 women; age: 23 years; BMI: 21.6kg/m^2.	Skin temperature (iButton).
Part 2: The importance of understanding the instruments: brown adipose tissue methodology			
5. A new personalized cooling protocol to activate brown adipose tissue in young adults.	Cross-sectional. Methodological study.	47 young adults (n=28 women); age: 22±2 years; BMI: 25.4kg/m^2.	Skin temperature (iButton), thermal perceptions (ASHRAE), brown adipose tissue (18F-FDG-PET/CT scan).
6. The impact of using BARCIST 1.0 criteria on quantification of brown adipose tissue volume and activity in three independent cohorts of adults.	Cross-sectional. Methodological study.	10 young lean adults; age: 25±2 years; BMI: 22.2±1.6kg/m^2. 10 young overweight/obese adults; age: 22±2 years; BMI: 29.0±3.4kg/m^2. 10 young overweight/obese adults; age: 42±6 years; BMI: 27.8±1.3kg/m^2.	Brown adipose tissue (18F-FDG-PET/CT scan).

7. Distribution of brown adipose tissue radiodensity in young healthy adults.	Cross-sectional. Methodological study.	125 young adults (n=82 women); age: 21.9±2.1 years; BMI 24.9±4.8kg/m^2.	Brown adipose tissue (18F-FDG-PET/CT scan).

Part 3: Brown adipose tissue and thermoregulation

8. Concurrent validity of supraclavicular skin temperature measured by iButtons and infrared thermography as a surrogate maker of brown adipose tissue.	Cross-sectional. Methodological study.	12 young adults (n=10 women); age: 21.9±2.1 years; BMI 23.5±4.8kg/m^2.	Skin temperature by iButton and by infrared thermography, Brown adipose tissue (18F-FDG-PET/CT scan).
9. Association of wrist and ambient temperature with cold-induced brown adipose tissue and skeletal muscle 18F-Fluorodeoxyglucose in young adults.	Cross-sectional.	96 young adults (n=65 women) 21.9±2.3 years; BMI 25.2±4.8kg/m^2).	Wrist skin temperature and the personal level of environmental temperature, brown adipose tissue and other tissues (18F-FDG-PET/CT scan).
10. The mediating role of brown adipose tissue and skeletal muscle measured by 18F-Fluorodeoxyglucose in the thermoregulatory system in young adults.	Cross-sectional. Mediation analyses.	96 young adults (n=65 women); age: 21.9±2.3 years; BMI 25.2±4.8kg/m^2.	Wrist skin temperature and the personal level of environmental temperature, brown adipose tissue and other tissues (18F-FDG-PET/CT scan).
11. Skin temperature response to a liquid meal intake is different in men than in women.	Cross-sectional.	104 young adults (n=68 women); age: 21.9±2.3 years; BMI 25.2±5.7kg/m^2.	Skin temperature (iButton), thermal perceptions (VAS), resting metabolic rate, body composition (DEXA).

Part 4: Clinical Implication

12. Evidence of high 18F-Fluorodeoxyglucose uptake in the subcutaneous adipose tissue of the dorsocervical area in young adults.	Cross-sectional.	133 young adults (n=68 women); age: 22±2 years; BMI 25±5kg/m^2.	Skin temperature (infrared thermography), brown and subcutaneous adipose tissue (18F-FDG-PET/CT scan).

13. Associations between cardiorespiratory and muscular fitness with 18F-Fluorodeoxyglucose uptake by brown adipose tissue and skeletal muscle in young adults.	Cross-sectional.	119 young adults (n=81 women); age: 21.9±2.1 years; BMI 25.0±4.8kg/m^2.	Cardiorespiratory fitness (VO2max), muscular fitness (Handgrip) and brown adipose tissue (18F-FDG-PET/CT scan).
14. Distribution of brown adipose tissue, the thermoregulatory system and, physical activity and fitness in metabolically healthy but overweight-obese adults: a case-control study.	Cross-sectional.	60 young adults (n=44 women). MHOO, age: 22.1±0.4 years; BMI 28.9±0.5kg/m^2. MUOO, age: 23.1±0.5 years; BMI 30.3±0.7kg/m^2.	Brown adipose tissue and other tissues (18F-FDG-PET/CT scan), skin temperature (iButtons), thermal perception (ASHRAE), cardiorespiratory fitness (VO2max), muscular fitness (Handgrip) and PA (accelerometers).

18F-FDG: 18F-Fluorodeoxyglucose; ASHRAE: American society of heating, refrigerating and air-conditioning engineers; BMI: body mass index; CT: computed tomography; DEXA: Dual-energy X-ray absorptiometry; MHOO: metabolically healthy but overweight-obese; MUOO: metabolically unhealthy but overweight-obese; NA: not applied; PA: physical activity; PET: positron emission tomography; VAS: visual analogue scale; VO2max: maximum volume of oxygen.

PART 1

The importance of understanding the instruments: skin temperature assessment

Temperatus ® software:
a new tool to efficiently
manage the massive

CHAPTER 2

BACKGROUND

An iButton is a temperature sensor of small dimensions (button-sized; 16x6 mm^2), low cost (~US50$), with a stable and autonomous system that measures temperature and records the data in a protected memory section[1]. iButtons were originally designed for monitoring ambient temperature during transport and storage of perishable items such as food[2], but its application in other fields has increased substantially during last years (https://www.maximintegrated.com/en/products/digital/ibutton.html).

There are different models of iButtons with different functionalities and features that depending on the goal could fit better than others. In the last 10 years, iButtons have been used as a tool to measure temperature in different fields, such as: (i) human thermal physiology [1,3], (ii) animal thermal physiology, for instance to record body temperature in koalas[4], small reptiles[5], or bovines [6]; (iii) weather stations as greenhouses or crops [7]; and (iv) land surface temperatures [2].

In 1937, Hardy and Du Bois[8] were the first to study the effect of different ambient temperatures over parameters of skin temperature in humans using the Hardy radiometer[9]. Afterwards, iButtons have been used to monitor the effect of anaesthesia-treatment[10], exercise[11], circadian-rythms[12], heat[13], and cold[3] on skin temperature parameters. During an experiment, participants can wear 4-8 [14] or even 26 or more iButtons attached to the skin[15]. iButtons have been extensively used in human physiology studies showing to be valid and reliable devices [1,11].

Currently, programming (switching on) the devices, downloading the data, and analysing the information registered by the iButtons are arduous, difficult, and highly time-consuming tasks for researchers and technicians. Nowadays, Maxim Integrated Products, Inc (unique manufacturer of iButtons) offers a free software (One-Wire Viewer, OWV) developed in Java, which at its present form has several limitations. For instance, OWV does not offer any possibility to analyse the amount of data generated by iButtons, hence there is not a specific software that allows users to automatically analyse this amount of data.

Taking this into account, we have developed the Temperatus® software with the main aim of making the task of programming, downloading, and analysing the massive amount of data generated by iButtons effortless, intuitive, time-efficient, and user-friendly.

HOW TO MANAGE IBUTTONS IN HUMAN PHYSIOLOGY EXPERIMENTS: FROM THE DESIGN TO THE DATABASE

Our research group conducted an exercise-based randomized controlled trial[16], and we used almost around 200 iButtons daily (DS-1922-L). Although OWV is a tool designed to manage iButtons, this software presents several restrictions making it inefficient with the tasks becoming time-consuming (Table 1).

Steps to design and use iButtons in a human experiment

Figure 1 shows a summary of all steps needed to conduct human physiological studies with iButtons (measurement of skin temperature parameters), and that can be applied to other fields of study. The user needs to decide a priori: (i) the number of iButtons required, (ii) the anatomical areas, and (iii) how to codify the iButtons for the data collection in the different areas. When the user has decided these features of study design, they can start the data collection of skin temperature. For instance, we have recently studied how the available equations to estimate the same parameter of skin temperature (e.g. mean, proximal, distal, etc.) can differ between them in different scenarios (warm vs. cold) [3]. In this experiment, we used 26 iButtons in 11 men[3].

Table 1: Main features and differences between One-wire view (OWV) and Temperatus®.

Main features	One-Wire View (OWV) software by Maxim Integrated Products, Inc	Temperatus® by the University of Granada
Free	✓	✗ 30 Days free licence
Intuitive	✗	✓
Alias	✗	✓
Positions	✗	✓
Calculator	✗	✓
Set of iButtons	✗	✓
Programme (overall)	✓	✓
Programme of 2 iButtons at the same time, automatically	✗	✓
Download (overall)	✓	✓
Download of 2 iButtons at the same time, automatically	✗	✓
Analysis of csv files of iButtons	✗	✓
Is it able to detect the battery of the iButtons?	✗	✗
Compatible with Windows system	✓	✓
Compatible with Mac OS	✗	✗
Reduction of the possibilities of a human mistake	✗	✓

With the OWV software, we would have spent ~30 seconds per iButton for programming and another ~30 seconds per iButton for downloading the information, a total time of ~26 minutes for a set of iButtons for every participant (~26 minutes multiplied by 11 participants = ~4.7 hours in total). In contrast, using Temperatus ® we spent ~20 seconds per 2 iButtons for programming and another ~20 seconds per 2 iButtons for downloading the information, a total time for ~8 minutes for a set of iButtons for every participant (~8 minutes multiplied by 11 participants = ~1.5 hours in total). Thus, Temperatus® drastically reduced the time needed to programme and download the iButtons. Temperatus® allows the user to programme 2 iButtons at the same time and synchronise the devices automatically with the hour of the user's computer, whereas OWV does not. To download the information using OWV, the user obtains a single .csv file for every iButton. Moreover, with OWV, they should have an advanced excel knowledge to

organise and process all the information generated by iButtons in order to prepare a final database. Since not all users have an advanced excel knowledge, we have developed Temperatus® in order to help researchers from all the world to manage all the information obtained from their iButtons experiments, see Figure 1.

TEMPERATUS® SOFTWARE

Temperatus® has been developed in a framework of human physiology laboratory, therefore, some of its features are prepared for human studies, albeit these features can be easily adapted to other fields.
From a technical point of view, Temperatus® has been developed using the Software Engineering standards, following the spiral model, where prototyping plays an important role. Using this developing technique, the software has been refined until reaching its latest form.

It has been developed as a desktop application for a single user and programmed in the Java language. Following the Model-view-controller pattern, the different packages and modules that compose the programme are arranged in a hierarchical fashion, which facilitates the incorporation of new requirements or functionalities and the modification of the existing ones. The user interface has been implemented using JavaFX and FXML (http://www.oracle.com/technetwork/java/javase/overview/javafx-overview-2158620.html) and designed focusing on its usability and following the design principles of user interfaces, integrating very simple, intuitive, and self-contained views.

It uses the H2 database (http://www.h2database.com) as database engine. It guarantees an efficient management of large volumes of data and it offers a reduced footprint, contributing to the application's running smoothness. Temperatus® integrates H2 database in an embedded way, so it is totally transparent for the user. Currently, there is an available free trial of 30 days that allows users to analyse their iButtons in http://profith.ugr.es/temperatus?lang=en.

MAIN FEATURES OF TEMPERATUS®

Before starting the data collection

This section describes the main features of Temperatus®: from connecting the iButtons to the PC, to obtaining a database file with all the information analysed. In order to facilitate the comprehension of the process, we have used a human study as an example in this article, in which we used 14 iButtons attached to the skin following the ISO-Standard organization[14], and we registered information at 1-minute intervals.

Connecting iButtons to the user's PC

Temperatus® has been designed to use the current hardware developed by Maxim Integrated Products, Inc. and several models of iButtons, see Table 2. The user needs a USB adaptor and a blue dot receiver to connect two iButtons to the PC at the same time.
https://www.maximintegrated.com/en/products/ibutton/ibuttons/blue_dot.cfm

Table 2: Models of iButtons devices compatible with Temperatus® software.

iButtons devices: DS1820, DS1822, DS1920, DS1921, DS1922, DS1923, DS2422, DS2438, DS2760, DS2762, DS18B20 and DS28EA00
Blue dot receivers: DS9490 and DS9481R

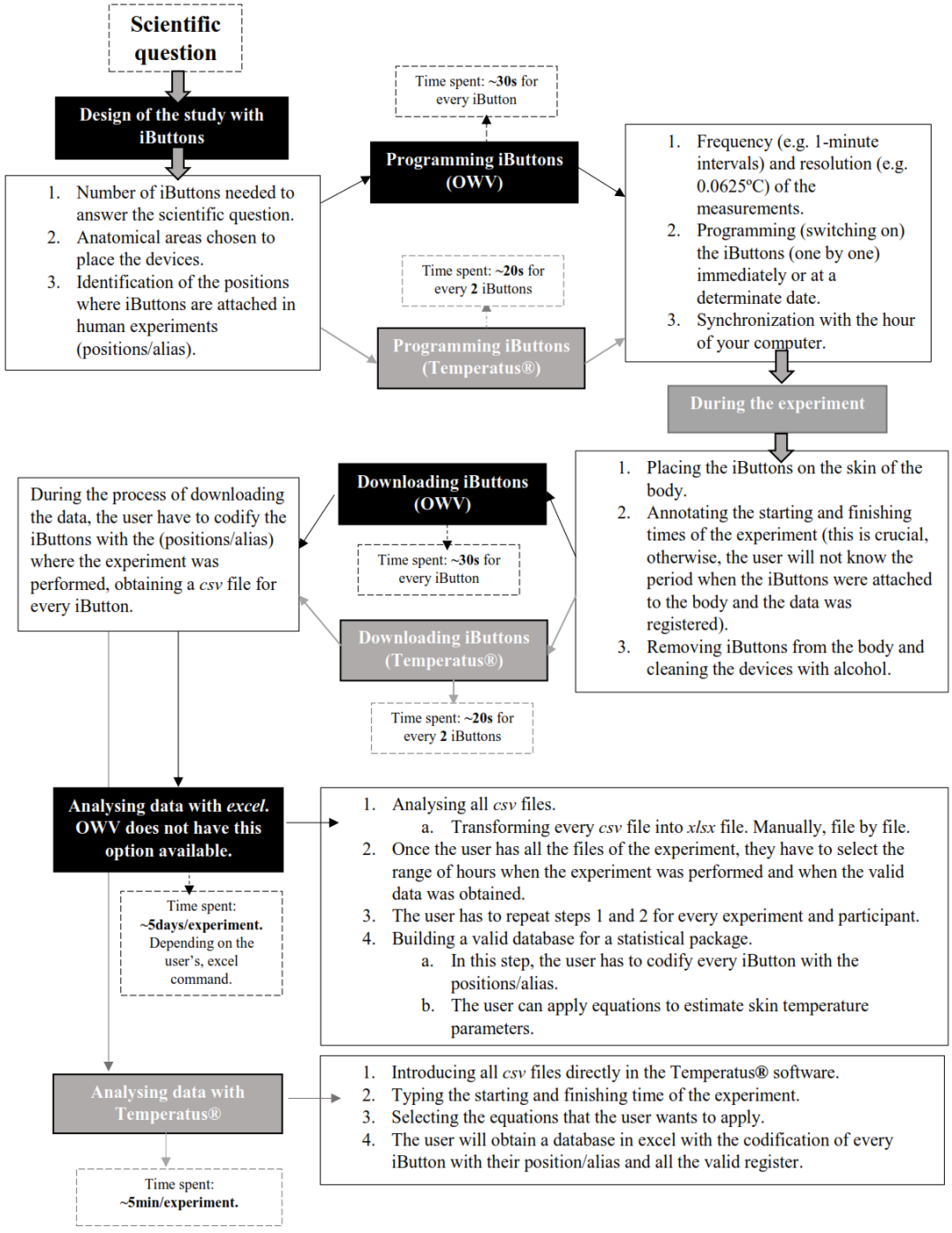

Figure 1. Summary of all the steps needed to use iButtons from the design of the experiment to obtaining the final database. These steps are summarised using the current tools for users [One-Wire Viewer (OWV) + excel work] and Temperatus® software, as a new tool to save time and facilitate the use of iButtons for users.

Aliasing and positioning: an easy way to encode the user's devices

The main aim for this feature is to create an individualized alias for every single iButton to help users to control and assign devices to different anatomical positions. Every single iButton has a single and unique long and difficult to read serial number on the case. The serial number is the best way to identify iButtons.Temperatus® shows the serial number of the iButton on the screen and gives the opportunity to encode the devices with a number, letter, or a word (alias). Once the alias has been assigned, it will appear on the screen along with its serial number when the iButton is connected (see Figure 2A). During the data collection, the user will normally write an alias or a code on the case of the device with a marker, which will help to recognise the position where the iButton was attached during the experiments (see Figure 2B). If this alias fades during the measurement, the user will be able to quickly know the alias of the iButton once it is connected to the PC.

Calculator

Temperatus® allows making mathematical calculations with the data registered. In human physiology experiments, iButtons are usually grouped to estimate mean, proximal, or distal skin temperatures, among others [3], and there are several equations to estimate the same parameters of skin temperature but that do not seem to measure exactly the same parameters [3]. The available equations to estimate those parameters of skin temperature included in the Temperatus® were previously studied [3]. Moreover, the user can introduce and create new equations or mathematical calculations, following the Boolean logic and using dots as decimal separator (Figure 3A).

Set of iButtons

Temperatus® allows using and managing as many iButtons as needed. For instance, if the user wants to create a set of 14 iButtons, they should select the number of iButtons (14 in this example) and the anatomical position for every device, for instance 1. Forehead; 2. Neck; 3. Left Chest, etc. The user can assign the equations to estimate the parameters of skin temperature to the set of iButtons (Figure 3B). For example, if the user creates 5 equations to estimate the mean skin temperature, they need to assign these equations to the predefined set of iButtons. If one or more equations are not possible to be calculated because, for example, one of the iButtons needed to estimate the temperature was not used, Temperatus® will code the cells of the database spreadsheet as #¡NUM!. As a result, the user will be able to easily identify which equation was not applied for this set of iButtons.

The features of "alias", "positions", "calculator", and "set of iButtons" are own features of Temperatus® that the OWV software does not include (see Table 1), and that makes the data collection much easier and time-efficient.

Using iButtons during the data collection

In this section, we show the improved features of programming and downloading iButtons during the data collection in comparison to the OWV software.

Programming iButtons

For a human thermal experiment, the user usually has to programme (switch on) more than one iButton and synchronise them with the hour of the PC simultaneously. However, if the user wants to programme more than one iButton, they must repeat the process of programming and synchronising as many times as iButtons they need, making this process maddening and time-consuming. Of note is that the probability of making a mistake in this process is quite high. In addition, the blue dot receivers are prepared to initialise two iButtons at the same time, but the OWV software is not able to apply this option available in the hardware (blue dot receivers).

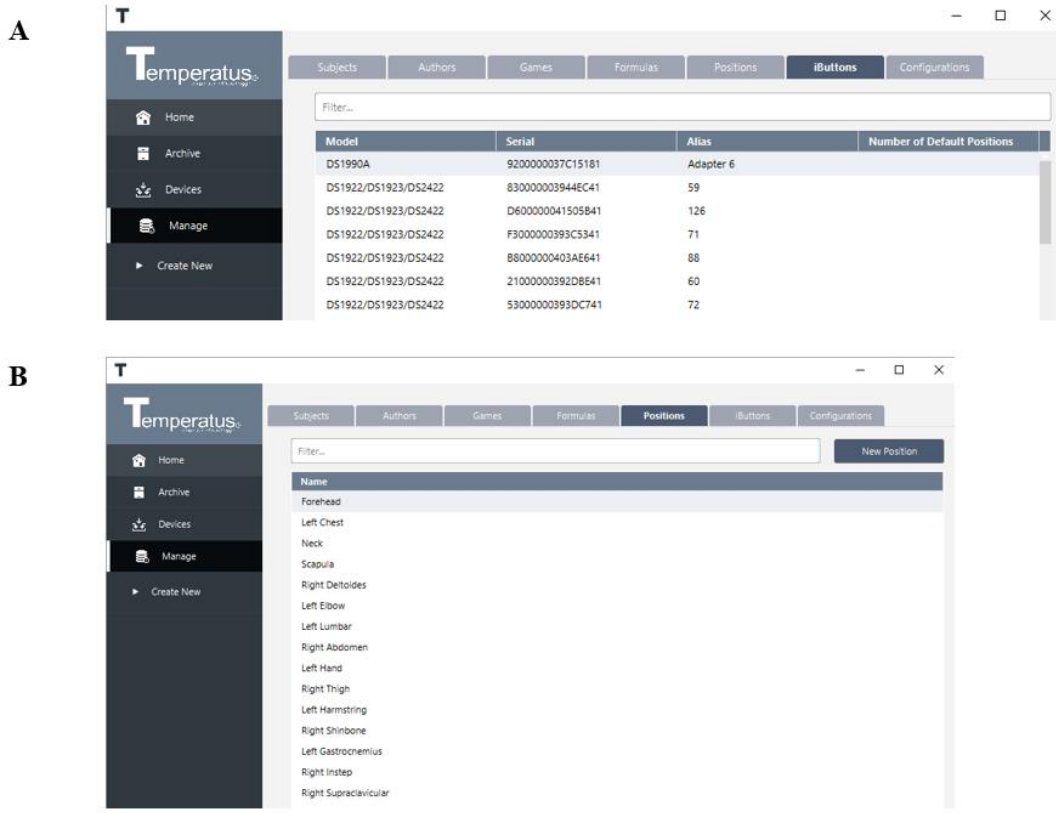

Figure 2. **A.** Alias codification for every iButton's serial number and blue dot receiver. **B.** Positions that can be created by users. In this example, the positions refer to anatomical points.

Temperatus® allows the user to programme the iButtons and save their configuration, so that it can be applied later to every single iButton. For instance, if the user wants to programme 14 iButtons, the user can select the frequency (e.g. 1 minute interval), resolution (e.g. 0.0625°C) and the moment to start recording data (immediately or a determinate date) and save the configuration. After that, the only thing that the user should do is affix the iButton (or two iButtons at the same time) to the blue dot receiver and connect the devices to the USB of the PC, as Maxim Integrated Products, Inc. established in their web page (https://www.maximintegrated.com/en/products/ibutton/ibuttons/blue_dot.cfm). After a few seconds, Temperatus® recognises the iButtons, and, in that moment. This feature saves a lot of time, and it ensures that all iButtons are programmed in the same way.

The OWV software does allow users to programme iButtons for next day, next month, or from one week to another, for instance ("determinate date" feature). Nevertheless, if the user needs to do that, the exact number of minutes that there is in one week must be introduced (e.g. 10080 minutes). Temperatus®, however, allows introducing the exact date and hour (format "dd/mm/yyyy hh:mm:ss") without the need to make any mathematical calculation of the number of minutes before starting the recording.

Downloading data from iButtons

Once the data recording is finished, it is necessary to extract the data from the iButton. In the OWV software, the user needs

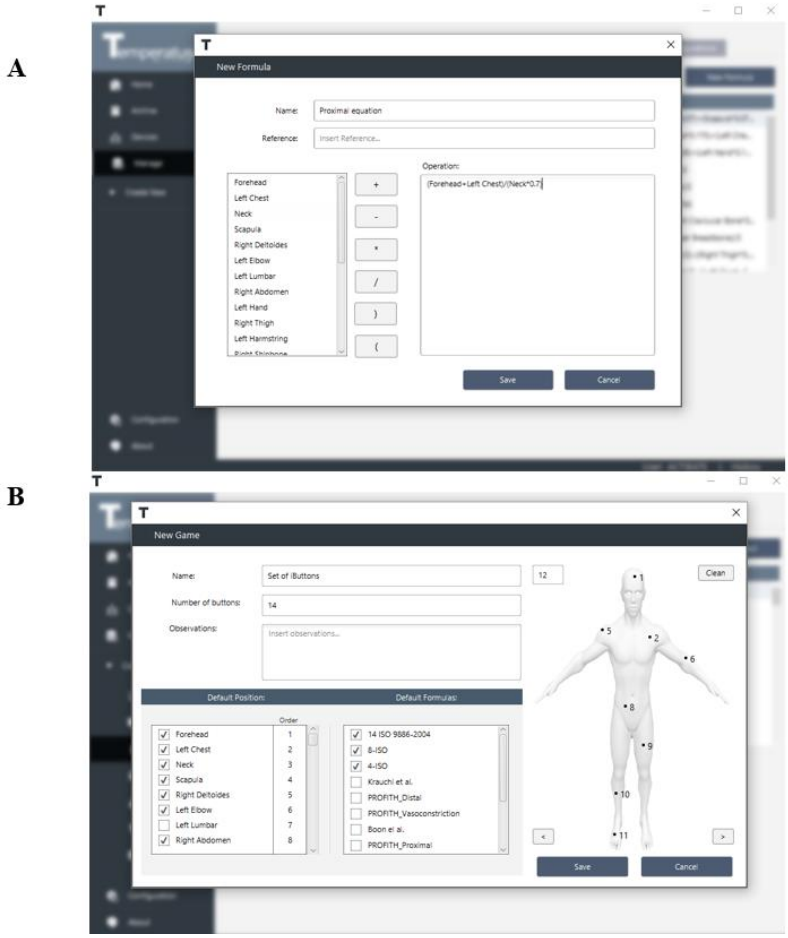

Figure 3. **A**. Calculator of Temperatus®, based on the anatomical positions that the user should create previously. **B.** Predefined set of iButtons. The user has to select the amount of iButtons and assign the positions and equations that they have created previously. Also, the user can define the default positions of every single iButton in a human body based on the alias codification.

to perform this task iButton by iButton. It is important to understand that the OWV software only allows users to download the data in csv or txt files. In contrast, the Temperatus® user does not need to repeat any manual step. The user must connect iButtons to the blue dot receivers in the PC and start downloading the data. Moreover, the user can choose to download the data in csv or xlsx files. We recommend users to save the data as csv files format if they are planning on analysing the data with Temperatus®, because this is the only file format that Temperatus® is prepared to analyse. In addition, Temperatus® downloads every data of iButton directly writing the alias automatically on the file, whereas with the OWV user has to write the code or alias manually on the csv file. This specific feature was thought to reduce the possible human mistake when files are renamed by hand and one-by-one. The features "programming" and "downloading" are improved features of the OWV software applied to Temperatus® to reduce human mistakes and reduce the time needed. The free trial available in http://profith.ugr.es/temperatus allows users to test these features for free and without a time limit. The feature for analysing iButtons is free for 30 days.

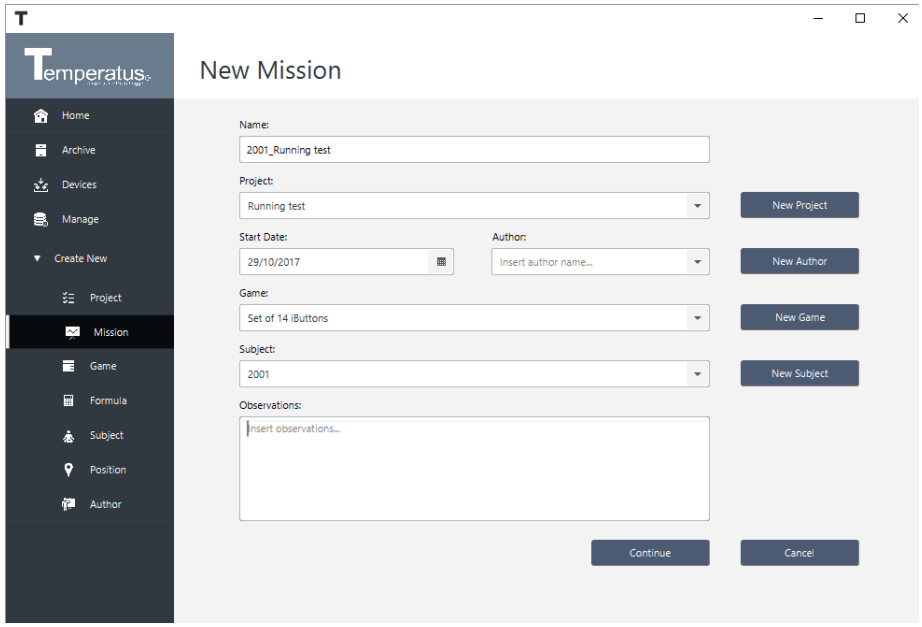

Figure 4. Mission tab to analyse iButtons. This screenshot shows the information the user should complete regarding (i) the name of the analysis, (ii) project, (iii) starting date of the analysis, (iv) author's name, (v) set of predefined iButtons, and (vi) identification.

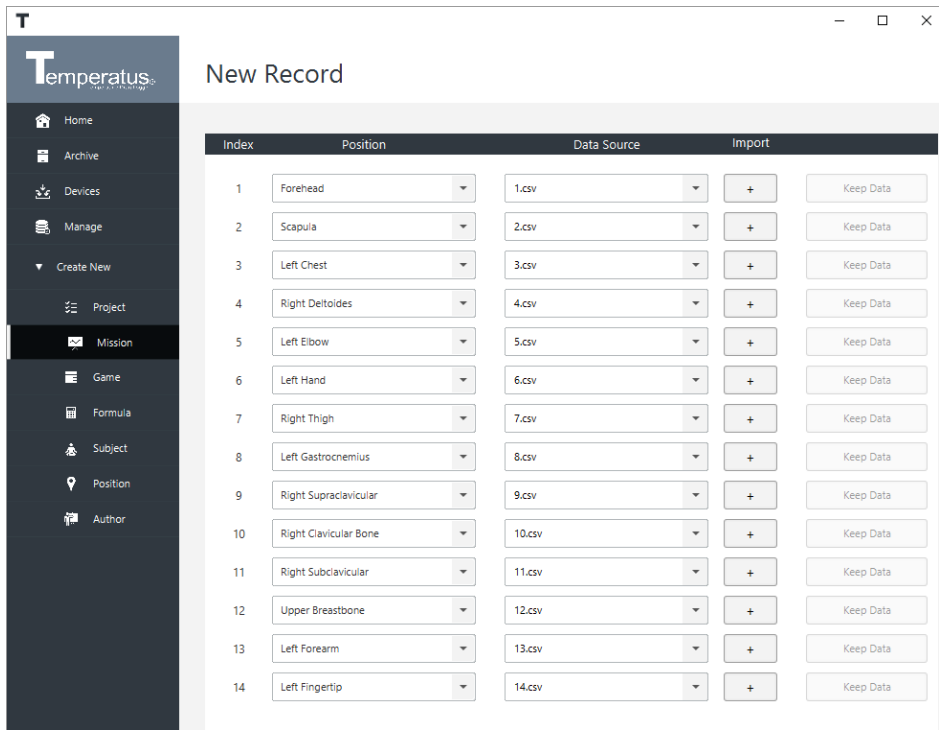

Figure 5. Importation of csv files to Temperatus® software. It is important to assign to every iButton the anatomical position where it was placed, because equations are based on the anatomical positions.

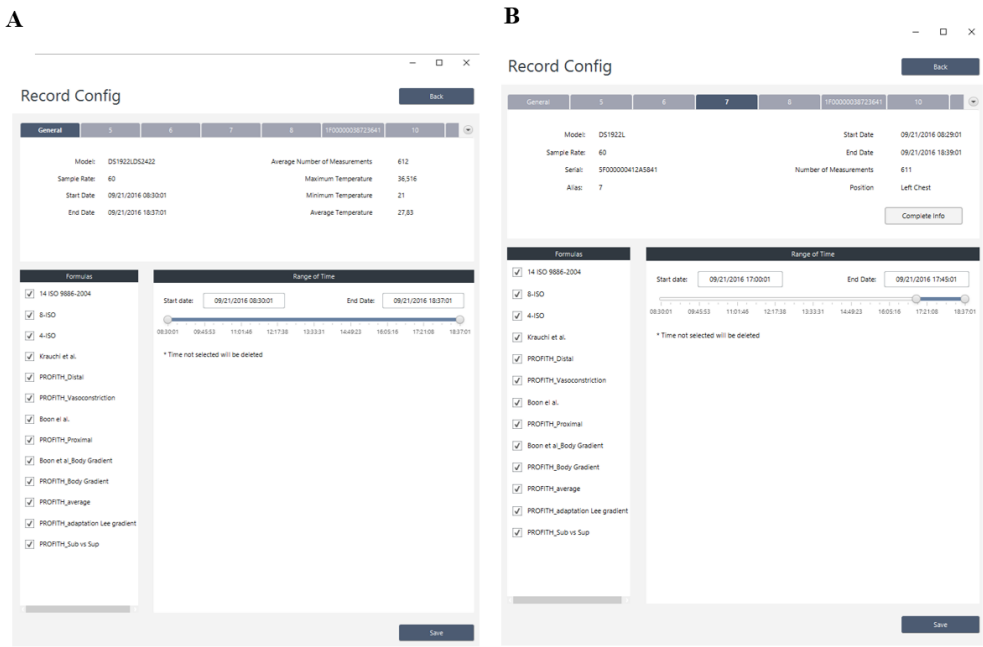

Figure 6. **A**. represents the whole iButtons time-line. **B**. The blue bar represents the period of time selected for analysis with Temperatus®, whereas, the rest of the period was deleted.

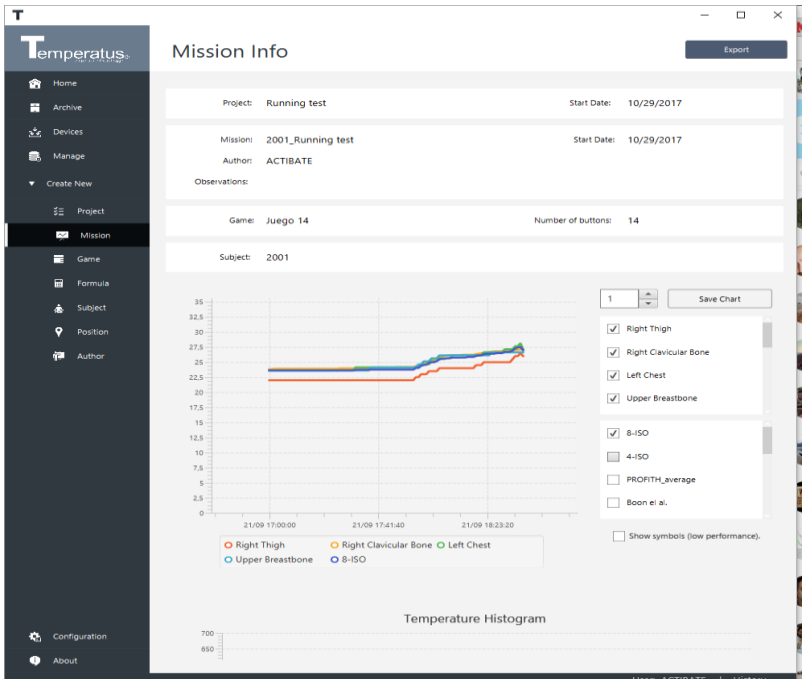

Figure 7. This screenshot represents the summary of the analysis with Temperatus®. The user can click on the positions or equations and they will appear in the chart (charts are possible to save as images .jpg or .png).

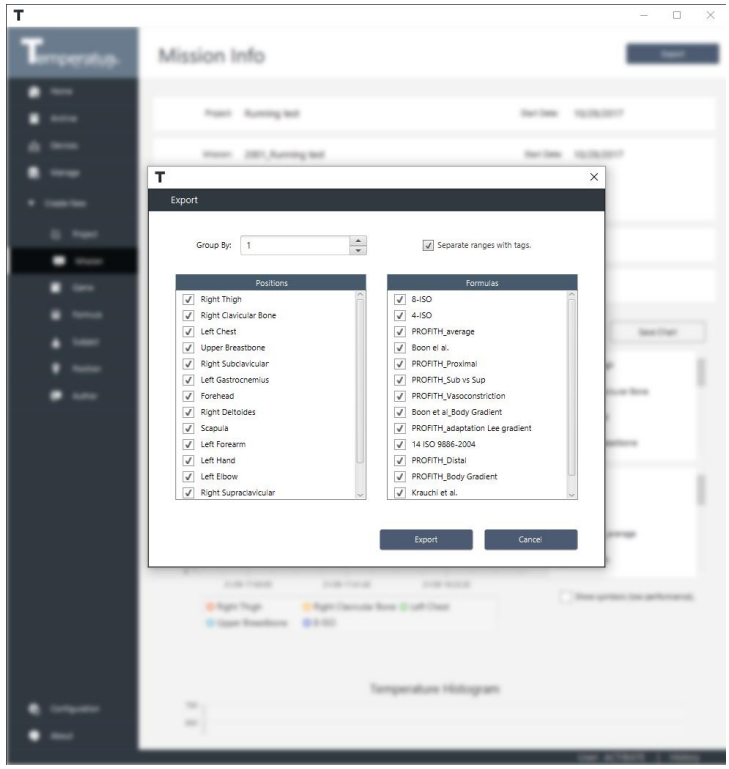

Figure 8. Export option: when the user clicks on "export", they will have the opportunity to download the data obtained from every single anatomical position and equations in .xlsx format. Also, the user will be able to group the data in intervals of 5 and 10 minutes.

	A	B	C	
1		Fri Feb 10 20:37:01 CET 2017	Fri Feb 10 20:38:01 CET 2	Fri
2	Forehead	33.852	33.977	
3	Left Chest	34.467	34.717	
4	Left Elbow	29.073	29.26	
5	Left Fingertip	24.408	26.845	
6	Left Forearm	25.426	28.986	
7	Left Gastrocnemius	29.64	30.139	
8	Left Hand	29.938	30.125	
9	Right Clavicular Bone	31.056	31.993	
10	Right Deltoides	33.034	33.471	
11	Right Subclavicular	29.884	32.13	
12	Right Supraclavicular	32.429	33.614	
13	Right Thigh	29.558	30.12	
14	Scapula	33.49	33.615	
15	Upper Breastbone	28.642	31.704	
16	14 ISO 9886-2004	#¡NUM!	#¡NUM!	#
17	4-ISO	#¡NUM!	#¡NUM!	#
18	8-ISO	31.650	31.958	3
19	Boon el al.	#¡NUM!	#¡NUM!	#
20	Boon et al_Body Gradient	#¡NUM!	#¡NUM!	#
21	Krauchi et al.	#¡NUM!	#¡NUM!	#
22	PROFITH_Body Gradient	-5.026	-4.854	
23	PROFITH_Distal	27.173	28.485	
24	PROFITH_Proximal	32.165	33.544	
25	PROFITH_Sub vs Sup	-2.545	-1.458	
26	PROFITH_Vasoconstriction	1.018	2.141	
27	PROFITH_adaptation Lee gradie	2.038	1.103	
28	PROFITH_average	30.522	30.554	3

Figure 9. This is a sample of the data treatment with Temperatus® in .xlsx file. The user will obtain a single xlsx file for every participant by every time point. #¡NUM! represents equations that are not possible to be calculated due to absence of data for one or more anatomical positions.

ANALYSING IBUTTON-GENERATED MASSIVE AMOUNT OF DATA: IT IS NOT AN IMPOSSIBLE MISSION

Adam et al.[4] declared that managing iButtons is a tedious and time-consuming task when large amounts of automatically collected data have to be processed. Brabyn et al.[2] used ArcGIS software using the 'ADD XY DATA' function and saved as a point shapefile. However, most of the human physiological laboratories used excel spreadsheets to manage and analyse all the data recorded by iButtons. Here, we provide an alternative way to analyse all the data of iButtons, in an easy, intuitive, and time-efficient way.

Temperatus® allows the user to analyse and process all data recorded by iButtons used on the same participant. This feature is named Mission (Figure 4). For instance, as explained before, we used a set of 14 iButtons in two participants in the same afternoon: one participant from 17:00:01 to 17:45:01 and the other from 19:02:01 to 19:58:01. We have to create as many missions as participants we want to analyse. Furthermore, Temperatus® permits users to save all the information regarding the participant's iButtons in a specific section of the software.

The user has to introduce all the information regarding the experiment that they consider important to add to the particular section. Also, they should select the "set of iButtons" used during the data collection, in order to apply the equations to estimate parameters of skin temperature for this analysis. Temperatus® allows users to analyse data that had been programmed by OWV. The only requirement is that all files need to be in csv format (Figure 5). It is important to be sure that csv files are in the corresponding anatomical position cells. Also, this software detects artefacts automatically. In human physiological experiments an artefact is considered when temperatures are higher than 45°C and lower than 10°C. The range of these artefacts can be defined by the user. During this analysis, the user has to introduce the valid period that they want to analyse. If there is a period of time during the measurements that must be deleted, the user has to select the period to exclude from the analysis in the timeline, and Temperatus® will only process the data for the selected periods. For instance, the 14 iButtons started the measurements at 8.A.M.; however, we only want to analyse the data recorded from 17:00:01 to 17:45:01 pm. With Temperatus®, we can select this range of time and analyse it (Figure 6A and B). This way, the user will automatically obtain the same valid range of time for all iButtons used in the same experiment and conditions.

During the analysis, the user can visualise a summary of their own data. They can download different charts, selecting the different positions of the iButtons or equations selected (Figure 7). The user can export all the analysed data to a database document, and they can decide to export the data as the lowest frequency available or as average of frequencies, for instance as an average of 5 minutes (Figure 8). At the end, the user will obtain a database for every experiment, which can be easily adapted to their statistical software package (Figure 9). This is a unique and one of the strongest features of Temperatus®. Also, Temperatus® is available to analyse the data of iButtons that were programmed and downloaded by the OWV software.

LIMITATIONS: FUTURE LINES

Although this first version of Temperatus® improves the work with the OWV software and adds new possibilities for analysing iButtons, it shows several limitations that should shortly be improved. To analyse more than one iButton at the same time, all data files must have the same day and hour of registration. OWV and Temperatus® are not compatible with operating systems such as Mac OS or Linux. Also, both softwares are not able to detect the battery life of each iButton, probably because iButtons do not have this option available.

CONCLUSIONS

To our knowledge, nowadays, Temperatus® is the unique alternative to the OWV software to programme, download, and the unique software that allows users to analyse massive amounts of data coming from different iButtons. This software has been developed, used, and validated in different laboratory conditions in the ACTIBATE study[16] at the University of Granada (Spain). Also, Temperatus® is being used in several human studies in the University of Cadiz (Spain), the University of Pamplona (Spain), and in the Leiden University Medical Center (The Netherlands).

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Differences between the most used equations in brown adipose tissue human studies to estimate parameters of skin temperature in young lean men

CHAPTER 3

BACKGROUND

In 1937, Hardy and Du Bois [1] studied the effect of different environment exposures (cold and heat) over parameters of skin temperature. Thereafter, parameters of skin temperature and thermal responses were studied as thermal-physiological responses to different stimulus such as exercise[2], anaesthesia-treatment[3], circadian-rythms[4], or heat[5] and cold[6] environment.

Since 2009, cold exposure is used in human studies as one of the main activators of brown adipose tissue (BAT) before performing a 18F-Fluorodeoxiglucose Positron emission tomography/computed tomography (18F-FDG-PET/CT)[7–12]. BAT is highly regulated by the sympathetic nervous system (SNS) increasing body temperature when mammals are exposed to cold[13]. Therefore, skin temperature measurements could play an important role as a possible indirect marker of BAT activity or volume [14–17].

To date, consensus about which equations are better to estimate parameters of mean, proximal, or distal skin temperature and body-gradients is non-existent. Moreover, whereas some human BAT studies [7,14,18–23] used different equations to estimate the same parameters of skin temperature, other studies did not report the equations used [15,24,25]. This fact hampers comparability between studies. The lack of knowledge about a set of validated equations or alternative instruments as "gold-standard" to measure skin temperature hinders comparisons between studies. Of note is that most of the human-studies used ibuttons (a valid [26,27] and reliable [27] tool) to measure skin temperature.

Most of the equations used in the cooling protocols before BAT activation were not validated (i.e. mean, proximal, distal skin temperature, and body gradients) against any "gold-standard", neither were most of the body-gradients used. To note is, however, that supraclavicular skin temperature [14,15,28] and a supraclavicular gradient [29] were validated against 18F-FDG-PET/CT and were postulated as an indirect measurement of BAT volume and activity. Sessler et al. [3] validated a peripheral skin temperature gradient (see Table 1) as a proxy of peripheral vasoconstriction measured by laser Doppler monitor. Furthermore, there are some thermophysiological models used to predict local skin temperature [25,30,31], yet these models need to be applied in the specific cooling protocols used in the BAT activation.

Taking into account the lack of consensus on which equation to use and the high discrepancy that exists across studies that measured skin temperature in response to cold exposure, we studied if the most used equations to estimate parameters of skin temperature in human BAT studies measure the same in young lean men.

MATERIAL & METHODS

A total of 11 men (23.4±0.5 years) took part in the present study. All participants were healthy, lean (fat mass: 19.9±1.2%) (see Table 2), non-smokers, and were not taking any medication that could have altered the cardiovascular or thermoregulatory responses to cold exposure. The study protocol and informed consent followed the Declaration of Helsinki (revision of 2013). This study was approved by The Human Research Ethics Committee of the University of Granada (n°924) and by the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada).

Previous conditions to the study day

The study was conducted between March and April 2016 in Granada (Southern Spain). Participants arrived at the research centre by bus or by car in fasted condition (at least 8 hours after their last meal), between 8 a.m. and 4 p.m. They were advised to refrain from any type of physical activity or exercise in the 48 hours prior to the study day. Additionally, participants were required not to drink alcoholic or caffeine-containing beverages in the 24 hours prior to the study day.

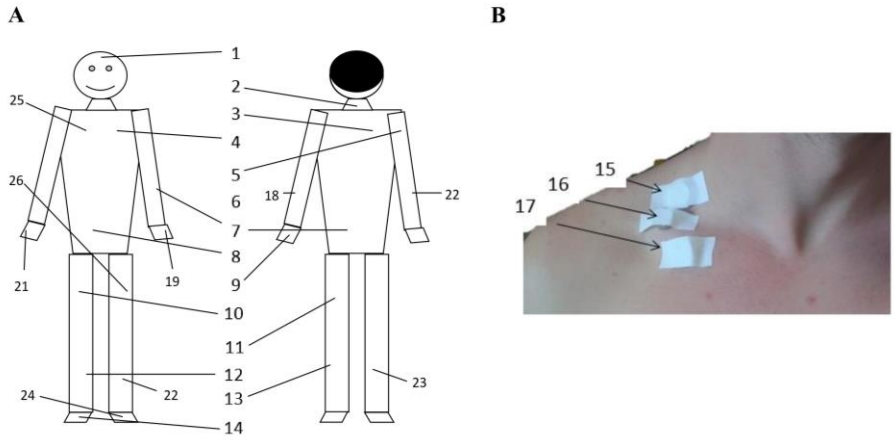

Figure 1. Anatomical position of 26 ibuttons. **A**: Distribution of the ibuttons over the body. **B**: Distribution of the ibuttons on the right clavicular sites.

Skin temperature registration

We measured skin temperature with 26 ibuttons [32] (DS-1922 L, Thermochron; resolution: 0.0625°C; Maxim, Dallas, USA). Ibuttons are valid and reliable devices to measure skin temperature in humans [26,27]. We attached the ibuttons to the skin with adhesive tape (Fixomull, Beiersdorf AG, Hamburg, Germany) on different body sites (see Figure 1) [14,26,32–36]. Skin temperature was recorded at 1-minute intervals. We reviewed which skin temperature equations were most used in human BAT studies [7,14,18–23], and selected 12 equations to estimate parameters of skin temperature including mean, proximal, and distal skin temperature (Table 1). Moreover, we calculated the gradient over forearms minus top of forefingers as a measure of peripheral vasoconstriction in the arms [37], as well as the heat loss capacity of the supraclavicular zone [29] and of the whole body through different body gradients [15]. Taking into account the number of ibuttons used in the present study (n=26), we computed 6 new equations using the highest number of ibuttons possible to estimate parameters of skin temperature (see PROFITH equations, Table 1). All data recorded by the devices and equations were analysed by the Temperatus software (http://profith.ugr.es/temperatus).

Table 2. Characteristics of the participants.

	Mean		SE
Age (years)	23.4	±	0.5
Body mass index (kg/m^2)	23.2	±	0.4
Fat free mass (kg)	58.2	±	8.8
Fat mass (kg)	13.8	±	0.9
Fat mass (%)	19.9	±	1.2
Bone mineral density (g/cm^2)	1.31	±	0.03

SE: Standard error.

Cooling protocol

Participants were barefoot and wore a short standardized T-shirt [clo-value: 0.20 [38,39]]. We controlled the room temperature (see Figure 2), and we avoided potential airflow in the room. The participants lay on a bed for 20 minutes in a warm room (warm period I, 22.7±0.2°C). Thereafter, participants were moved into a cold room (19.4±0.1°C) where we applied a cooling protocol until shivering occurred (Figure 2). In the cold room, the participants lay on a bed for 15 minutes, after which they were equipped with a temperature-controlled water circulation-cooling vest (Polar Products Inc., Ohio, USA) [40]. The cooling vest covered the individuals' clavicular region, as well as the chest,

abdomen, and back. The water temperature started at 17°C and decreased progressively (~1°C) every 10 minutes until shivering occurred (cold period mean time: 92.5±8.7 minutes; water mean temperature of shivering: 8.1±2.41°C, see Figure 2). We determined shivering both visually and by asking the participants if they were experiencing shivering. Shivering was confirmed by EMG in 6 participants. Once shivering was determined, the participants returned to the warm room (23.2±0.2°C) and lay on the bed for another 20 minutes (warm period II) without the cooling vest. The participants were not allowed to move on the bed, read or watch a film, or to be covered by a blanket or a sheet. At the end of the study day, we measured body composition by Dual Energy X-ray Absorptiometry scan (HOLOGIC, QDR 4500W).

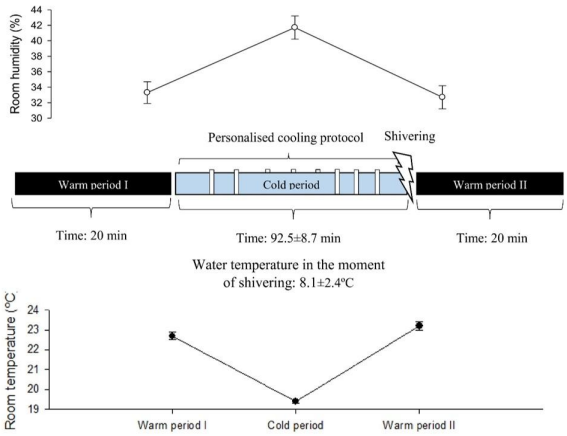

Figure 2. Cooling protocol. Each vertical white bars represents a decrease of the water temperature of the cooling vest. Upper graphic represents mean room humidity during warm period I, cold period, warm period II. Lower graphics represents mean room temperature during warm period I, cold period, warm period II.

Statistical Analysis

Data are presented as mean and standard error. The skin temperature data were taken as average in the last five minutes of the warm period I (before initializing cooling protocol), in the last five minutes of the warm period II, and in the five minutes prior to shivering (cold period). We excluded the data from the equations in the analysis when at least one ibutton failed during the measurements (see Table 1). We analysed differences across the study equations using analysis of variance (ANOVA) with Bonferroni adjustments for post-hoc comparisons, by periods (warm period I, cold period, and warm period II). We compared mean differences of skin temperature across temperature conditions, using ANOVA for repeated measurements. All the analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set to <0.05.

RESULTS

Warm period I

Figure 3 shows the mean skin temperature (A), proximal skin temperature (B), distal skin temperature (C), body temperature gradient (D), supraclavicular temperature gradient (E), and temperature gradients as a proxy for upper (left and right arm) and lower (left and right leg) peripheral vasoconstrictions (F) in the last five minutes of warm period I as estimated with the equations used in literature (Table 1). ANOVA showed differences across mean skin temperature equations (overall P<0.001, Figure 3A). The post-hoc analysis showed significant differences in mean skin temperature using the equation reported by 4-ISO [41] compared with 14-ISO [41] (mean difference 1.35°C; 95% confidence interval: 0.12°C-2.57°C; P=0.022) and between the equation reported by Boon et al. [15] and 8-ISO [41] (-1.83°C; -3.02°C- -0.64°C; P<0.001), 4-ISO (-2.15°C; -3.35°C- -0.96 °C; P=0.001), and PROFITH (-1.40°C; -2.67°C- -0.14°C; P=0.021).

Similarly, significant differences were observed across proximal skin temperature equations (overall P<0.001, Figure 3B). The post-hoc analysis revealed significant differences between the equation reported by Schellen et al. [32] compared to Boon et al. [15] (mean difference 1.03°C; 95% confident interval: 0.13°C-1.93 °C; P=0.014), Kräuchi et al. [36] (0.90°C; 0.01°C-1.81°C; P=0.048), and van Marken Lichtenbelt et al.

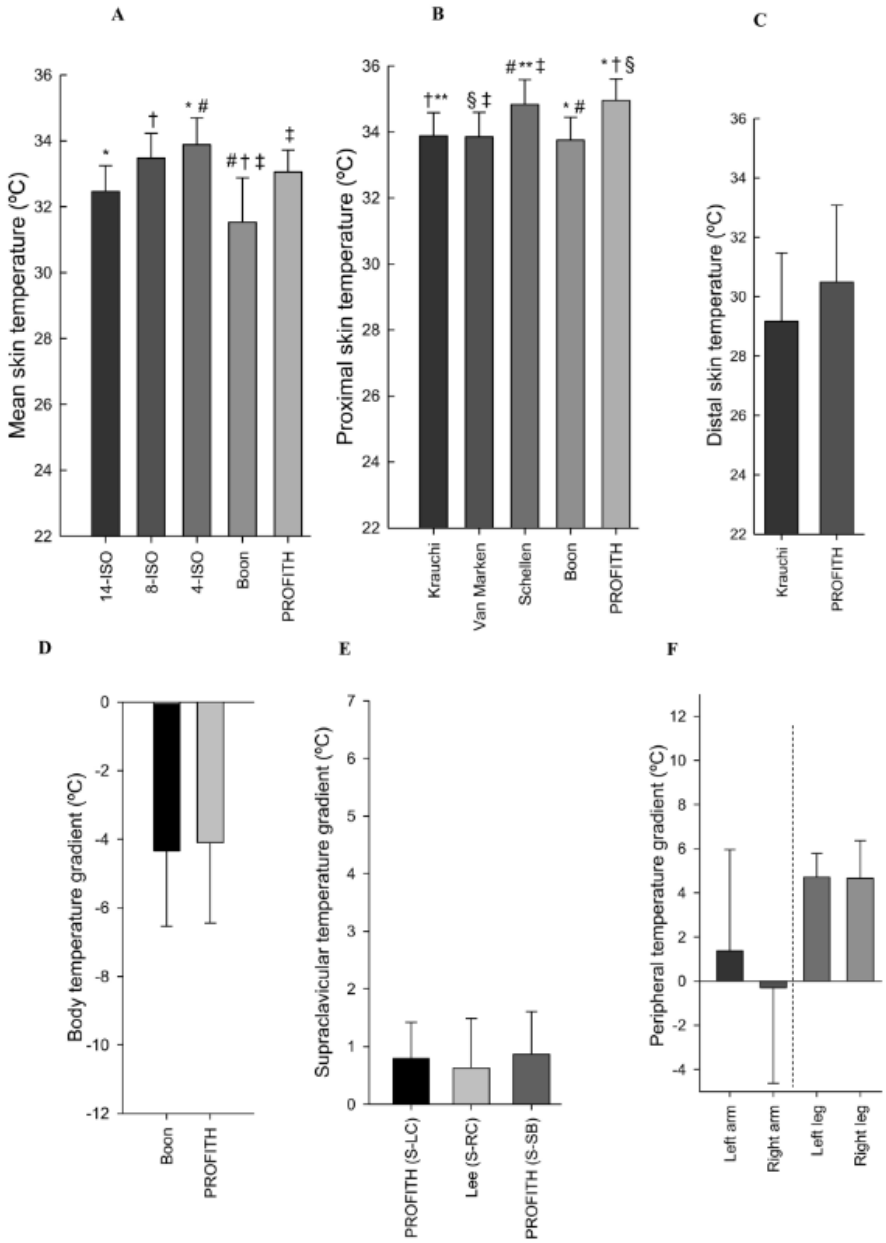

Figure 3. Measures of skin temperature at the last five minutes of the first warm period as estimated with the equations used in the respective references. A: Mean skin temperature: *P=0.022: 14-ISO vs. 4-ISO; †P<0.001: 8-ISO vs. Boon; #P=0.001: 4-ISO vs. Boon; ‡P=0.021: Boon vs. PROFITH. B: Proximal skin temperature: †P=0.015: Krauchi vs. PROFITH; **P=0.048: Krauchi vs. Schellen; §P=0.011: Van Marken vs. PROFITH; ‡P=0.036: Van Marken vs. Schellen; #P=0.014 Schellen vs. Boon; *P=0.004: Boon vs. PROFITH. C: Distal skin temperature: P= 0.222: Krauchi vs PROFITH. D: Body temperature gradients: P= 0.931: Boon vs PROFITH. E: Supraclavicular temperature gradients: (S: Supraclavicular; LC: Left Chest; RC: Right Chest; SB: Subclavicular); All P=1.000. F: Peripheral temperature gradient: All P=1.000. Data are mean and standard error.

[26] (0.93°C; -0.82°C-1.84°C; P=0.036). We also observed significant differences between PROFITH and Boon et al. [15] (1.16°C; 0.26°C-2.06°C; P=0.004), Kraüchi et al. [36] (1.03°C; 0.13 °C-1.93°C; P=0.015), and van Marken

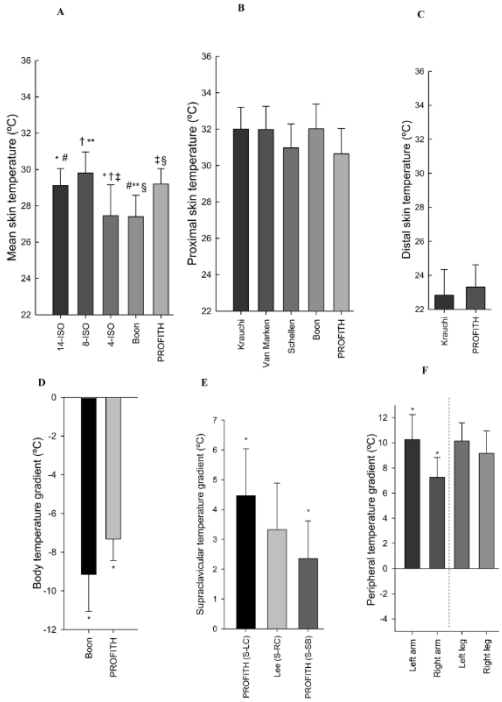

Figure 4. Measures of skin temperature at the last five minutes of the cold period as estimated with the equations used in the respective references. A: Mean skin temperature: *P=0.025: 14-ISO vs. 4-ISO; #P=0.027: 14-ISO vs. Boon; †P≤0.001: 8-ISO vs. 4-ISO; **P=0.001: 8-ISO vs. Boon; ‡P=0.022: 4-ISO vs. PROFITH; §P=0.024 Boon vs. PROFITH. B: Proximal skin temperature: All P≥0.123. C: Distal skin temperature: P=0.438. D: Body temperature gradients: *P=0.010 Boon vs. PROFITH. E: Supraclavicular temperature gradients: (S: Supraclavicular; LC: Left Chest; RC: Right Chest; SB: Subclavicular) *P=0.006; PROFITH (S-LC) vs. PROFITH (S-SB). F: Peripheral temperature gradient: *P= 0.001; Left Arm vs. Right Arm. Data are mean and standard error.

Lichtenbelt et al. [26] (1.07°C; 0.16°C-1.96°C; P=0.011).

There were no significant differences between the equations used to estimate distal skin temperature (Figure 3C), body (Figure 3D), and supraclavicular (Figure 3E) and peripheral (Figure 3F) temperature gradients (all P≥0.1).

Cold period

Figure 4 shows the mean skin temperature (A), proximal skin temperature (B), distal skin temperature (C), body temperature gradient (D), supraclavicular temperature gradient (E), and temperature gradients as a proxy for upper (left and right arm) and lower (left and right leg) peripheral vasoconstrictions (F) in the last five minutes of the cold period as estimated with the various equations used in literature (Table 1). Differences across mean skin temperature equations were found in warm room conditions (overall P<0.001, Figure 4A). The post-hoc analysis showed significant differences between the equation reported by Boon et al. [15] and 14-ISO [41] (mean difference -1.71°C; 95% confident interval: -3.30°C- -0.12°C; P=0.027), 8-ISO (-2.36°C;-3.90°C- -0.80°C; P=0.001), and PROFITH (-1.79°C; -3.44°C- -0.15°C; P=0.024). Similarly, there were significant differences between 4-ISO [41] and 14-ISO [41] (-1.73°C ;-3.32°C- -0.14°C; P=0.025), 8-ISO [41] (-2.37°C; 0.83°C- -3.93°C; P≤0.001), and PROFITH (-1.81°C; -3.45°C- -0.16 °C; P=0.022).

There were no significant differences across equations used to estimate proximal (P=0.123, Figure 4B) and distal skin temperature (P=0.438, Figure 4C). There were, however, significant differences between the equations used to estimate the body temperature gradient (-1.82°C; 95% confident interval: -0.82°C – -2.83°C; P=0.01, Figure 4D). Similarly, there were differences between the method to estimate supraclavicular temperature gradient zone when the skin temperature of the left chest zone was used instead of the right subclavicular zone (2.10°C; 0.51°C-3.70°C; P=0.006, Figure 4E), as well as between the left and right gradient of the arms to estimate peripheral temperature gradients (3.01°C; -0.97° C –5.04°C; P=0.001, Figure 4F).

Warm period II

Figure 5 shows the mean skin temperature (A), proximal skin temperature (B), distal skin temperature (C), body temperature gradient (D), supraclavicular temperature gradient (E), and temperature gradients as a proxy for upper (left and right arm) and lower (left and right leg) peripheral vasoconstriction (F) in the last five minutes of warm period II as estimated with the various equations used in literature (Table 1). Just as in warm period I, differences were found across mean skin

temperature equations (overall P<0.001,

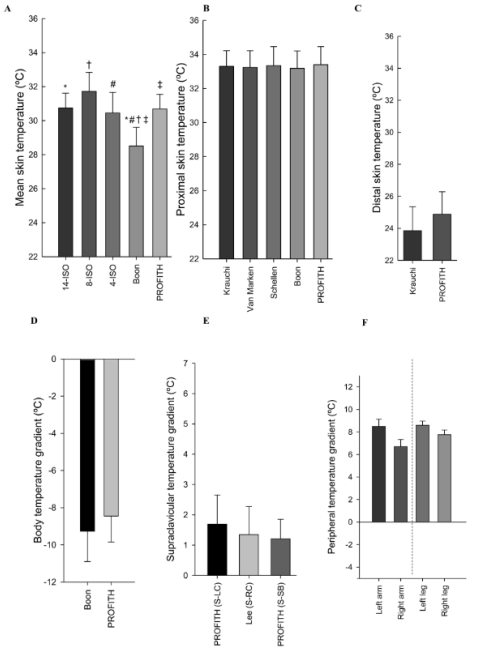

Figure 5. Measures of skin temperature at the last five minutes of the second warm period as estimated with the equations used in the respective references. A: Mean skin temperature: *P≤0.001: 14-ISO vs. Boon; †P≤0.001: 8-ISO vs. Boon; #P=0.002: 4-ISO vs. Boon; ‡P=0.001: Boon vs. PROFITH. B: Proximal skin temperature: All P=1.000. C: Distal skin temperature: P=0.113. D: Body temperature gradients: P=0.252. E: Supraclavicular temperature gradients: All P≥0.575. F: Peripheral temperature gradient: All P=1.000. Data are mean and standard error.

Figure 5A). The post-hoc analysis showed significant differences between the equation reported by Boon et al. [15] and 14-ISO [41] (mean difference -2.18°C; 95% confident interval: -3.56°C- -0.79°C; P≤0.001), 8-ISO (-3.10°C; -4.45°C- -1.76°C; P≤0.001), 4-ISO (-1.87°C; -3.21 °C- -0.52 °C; P=0.002), and PROFITH (-2.12°C; -3.55°C- -0.70°C; P=0.001). There were no significant differences (all P≥0.1) between the equations used to estimate proximal (Figure 5B) and distal skin temperature (Figure 5C), as well as body (Figure 5D), supraclavicular (Figure 5E), and peripheral (Figure 5F) temperature gradients.

DISCUSSION

Cold activates human BAT, which produces heat. Skin temperature is an indirect measure to monitor how the body reacts to cold. The present study analysed the impact of the most used equation in BAT-human studies to estimate parameters of skin temperature in warm and cold room conditions in young lean men. We observed differences across equations to measure the same parameters of skin temperature in warm and cold room conditions, which hamper comparisons across studies.

Mean skin temperature

The equation reported by ISO STANDARD 9886:2004 using 14 ibuttons (14-ISO) [41] is the most commonly used equation in BAT-related studies [7,14,18–23], yet it has been used with substantial modifications [15,24,25]. Furthermore, studies report values of mean skin temperature without providing information on how the calculations were made [12,17,29], which hampers between-study comparisons. Mean skin temperature estimated with 14-ISO was similar to that estimated using 26 ibuttons (PROFITH equation, see Table 1), which suggests that the 14-ISO equation covers the most important body sites. The ISO STANDARD 9886:2004 suggested another set of 8 ibuttons (8-ISO) [41] to measure mean skin temperature. However, the 8-ISO slightly overestimates temperature in both warm and cold conditions, at least when compared with the other equations used in this study.
Similarly, the equation based on 4 ibuttons (4-ISO) [41] overestimates mean temperature in warm room conditions and underestimates mean temperature in cold conditions compared to 14-ISO. The anatomical sites used in 4-ISO may partially explain the observed differences. For instance, the temperatures of the shin bone and the hand decreased after cold exposure [14] probably due to peripheral vasoconstriction, while these two anatomical zones contribute 50% of the estimated mean temperature in the equation 4-ISO. Based on these findings, we suggest using the 14-ISO equation to measure mean skin temperature because (i) it is the most used equation in BAT-human studies, (ii) it is supported by the International Standard Organization, and (iii) the outcome of temperature is practically the same when it is compared with an equation with a higher number of ibuttons.

Table 1. Equations used to estimate parameters of skin temperature.

Outcome	Reference	iButtons (n)	Anatomical positions. Figure 1	Participants (n)	Equations
Mean skin temperature	14 ISO 9886-2004 [41] (14-ISO)	14	From 1 to 14	9	(Forehead*0.07)+(Neck*0.07) +(Right Scapula*0.07)+(Left Chest*0.07)+(Right Deltoid*0.07)+(Left Elbow*0.07)+(Right Abdomen*0.07)+(Left Hand*0.07)+(Left Lumbar *0.07)+(Right Thigh*0.07)+(Left Hamstring*0.07)+(Right Shinbone*0.07)+(Left Gastrocnemius*0.07)+(Right Instep*0.07)
	8 ISO 9886-2004 [41] (8-ISO)	8	1,3,4,5,6,9, 10,13	10	(Forehead*0.07)+(Right Scapula*0.175)+(Left Chest*0.175)+(Right Deltoid*0.07)+(Left Elbow*0.07)+(Left Hand*0.05)+(Right Thigh*0.19)+(Left Gastrocnemius*0.2)
	4 ISO 9886-2004 [41] (4-ISO)	4	2,3,9,12	10	(Neck*0.28)+(Right Scapula*0.28)+(Left Hand*0.16)+(Right Shinbone*0.28)
	Boon et al. [15] (Boon)	5	10,16,8,9,14	10	(((Right Thigh*0.383)+(Right Clavicular *0.293)+(Right Abdomen*0.324))+ ((Left Hand+Right Instep)/2))/4)
	PROFITH	26	From 1 to 26	8	(Forehead+Neck+Right Scapula+Left Chest+Right Deltoid+Left Elbow+Right Abdomen+Left Hand+Left Lumbar +Right Thigh+Left Hamstring+Right Shinbone+Left Gastrocnemius+Right Instep+Right Supraclavicular +Right Clavicular +Right Subclavicular +Left Forearm+Left top of forefinger+Right Forearm+Right top of forefinger +Left Shinbone+Right Gastrocnemius+Left Instep+Right Chest+Left Thigh)/26
Proximal skin temperature	Kräuchi et al. [36] (Kräuchi)	4	1,10,17,8	10	(Forehead*0.093)+(Right Thigh*0.347)+(Right Subclavicular*0.266)+(Right Abdomen*0.294)/4
	van Marken Lichtenbelt et al. [26] (Van Marken)	3	10,17,8	10	(Right Thigh*0.383)+(Right Subclavicular*0.293)+(Right Abdomen*0.324)
	Schellen et al. [32] (Schellen)	4	3,7,4,8	11	(Right Scapula+Left Lumbar +Left Chest+Right Abdomen)/4
	Boon et al. [15] (Boon)	3	10,16,8	10	(Right Thigh*0.383)+(Right Clavicular*0.293)+(Right Abdomen*0.324)
	PROFITH	5	8, 25, 4, 3,2	11	(Right Abdomen+Right Chest+Left Chest+Right Scapula+Neck)/5

Distal skin temperature	Kräuchi et al. [36] (Krauchi)	2	9, 14	11	(Left Hand+Right Instep)/2
	PROFITH	6	1, 19, 21, 14, 24, 9	11	(Forehead+Left top of forefinger +Right top of forefinger +Right Instep+Left Instep+ Left Hand)/6
Body temperature gradient	Boon et al. [15] (Boon)	5	9,14,10,16,8	10	[(Left Hand+Right Instep)/2]- [(Right Thigh*0.383)+(Right Clavicular*0.293)+(Right Abdomen*0.324)]
	PROFITH	11	1, 19, 21, 14, 24, 9,8,25,3,2	11	[(Forehead+Left top of forefinger +Right top of forefinger +Right Instep+Left Instep+ Left Hand)/6]-[(Right Abdomen+Right Chest+Left Chest+Right Scapula+Neck)/5]
Supraclavicular temperature gradient	PROFITH: (S-LC)	2	15, 4	11	(Right Supraclavicular(S)-Left Chest(LC))
	Lee et al. [29] (Lee S-RC)	2	15, 25	11	(Right Supraclavicular(S)-Right Chest (RC))
	PROFITH: (S-SB)	2	15, 17	11	(Right Supraclavicular(S)-Right Subclavicular(SB))
Peripheral temperature Gradient	Sessler et al. [3] Right arm	2	20, 21	11	(Right Forearm-Right Top of forefinger)
	PROFITH: Left arm	2	18,19	11	(Left Forearm-Left Top of forefinger)
	PROFITH: Right Leg	2	23,14	11	(Right Gastrocnemius-Right Instep)
	PROFITH: Left Leg	2	13,24	11	(Left Gastrocnemius-Left Instep)

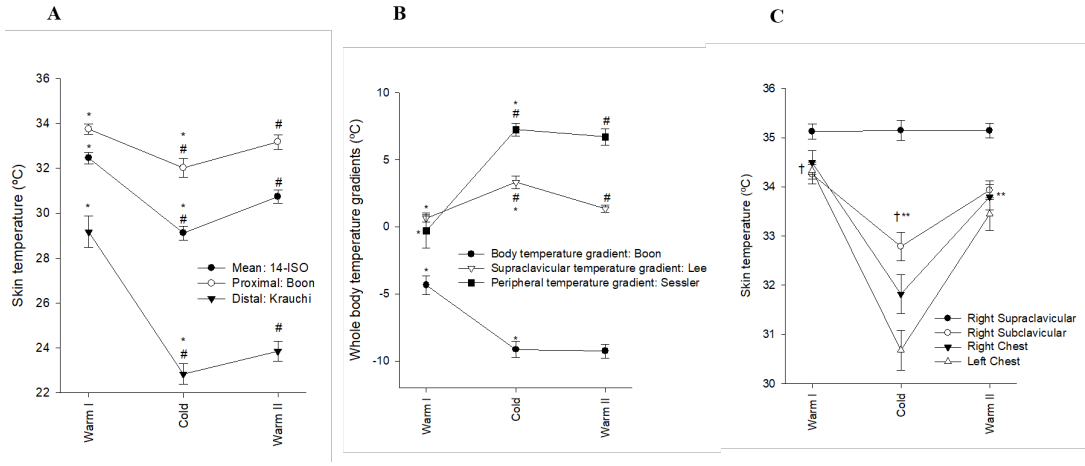

Figure 6. Suggested equations to measure different parameters of skin temperature during warm and cold exposures. A: Mean skin temperature (14-ISO): *P≤0.001: Warm I vs. Cold; #P≤0.001: Cold vs. Warm II; #P=0.002. Proximal skin temperature (Boon): *P≤0.001: Warm I vs. Cold; #P≤0.001: Cold vs. Warm II. Distal skin temperature (Krauchi): *P≤0.001: Warm I vs. Cold; #P≤0.001: Cold vs. Warm II. B: Body temperature gradient (Boon):*P≤0.001: Warm I vs. Cold. Supraclavicular temperature gradient (Lee):*P≤0.001: Warm I vs. Cold; #P≤0.001: Cold vs. Warm II. Peripheral temperature gradient (Sessler): *P≤0.001: Warm I vs. Cold. C: Raw data of all anatomic points integrated in the equations to estimate supraclavicular temperature gradients: all †P≤0.001: Warm I vs. Cold; all**P≤0.001: Cold vs. Warm II. No significant differences were found in the supraclavicular skin temperature across the exposures. Data are mean and standard error.

Proximal skin temperature

We did not observe differences between the equations used to estimate proximal skin temperature during cold exposure or during the warm period II. On the other hand, there were differences between the study equations during the warm period I. The equations reported by Kräuchi et al. [36] and Schellen et al. [30] used four ibuttons while the equations reported by van Marken Lichtenbelt et al. [26] and Boon et al. [15] used three ibuttons. The equations reported by both van Marken Lichtenbelt et al. [26] and Boon et al. [15] are based on the same anatomical points. However, Boon et al. [15] used the clavicular zone whereas van Marken Lichtenbelt et al. [26] used the subclavicular zone (Table 1). These equations showed a similar decrease of the mean temperature after cold exposure: -1.72±0.85°C [15] and -1.87±0.76°C [26], respectively. To assess proximal skin temperature, we suggest the equation reported by Boon et al. [15] as it includes a button at the clavicular site which is close to BAT deposits. In addition, the outcome of proximal temperature could be more representative from a body reaction to cold than other equations.

Distal skin temperature

To measure distal skin temperature, the majority of BAT-related studies [7,14,18–23] used the equation reported by Kraüchi et al. [36] (2 ibuttons placed on the left hand and right instep). Indeed, this equation is one of the easiest to estimate distal skin temperature. We did not observe differences between distal skin temperature measured by this and other equations with a higher number of distal anatomical points (6 ibuttons, see PROFITH, Table 1) in warm or cold conditions (all P≥0.113). In addition, the 14 anatomical points recommended by ISO STANDARD 9886:2004 [41] include the anatomical positions used by the equation reported by Kraüchi et al. [36]. Thus, under these study conditions, the equation reported by Kraüchi et al. [36] is a valid choice to estimate distal skin temperature. Moreover, two distal ibuttons (hand and feet) can measure the same as other equations with a higher number of ibuttons (i.e. 6 devices in PROFITH equation, see Table 1).

Whole body temperature gradients

The heat loss capacity of the body can be estimated as the gradient between distal and proximal skin temperature. Such gradients have been used in various fields including circadian rhythm [34,36,42], anaesthesia [24], exercise [27,43,44], and following BAT activation [7,14,18–23]. We observed no differences between the gradients calculated by the equation reported by Boon et al. [15] (5 ibuttons) and other equations with a higher number of anatomical points (11 ibuttons, see PROFITH, Table 1). Therefore, we suggest to use the equation reported by Boon et al. [15] as it obtains the same outcome of temperature with a lower number of ibuttons than other equations.

Another interesting gradient that has recently been used in the field of BAT research is the supraclavicular temperature gradient proposed by Lee et al. [16]. This gradient is based on the difference between the temperature of the right supraclavicular fossa and right upper chest and aims to estimate BAT heat loss capacity during or after a cold exposure. Yoneshiro et al. [12] and Chondronikola et al. [17] used the same gradient albeit at the left side of the body. The importance of the side of the body is unknown and further studies are warranted. Nevertheless, we observed differences when the supraclavicular temperature gradient is calculated on the right or the left side, as well as on the chest or the subclavicular zone. We propose to use the supraclavicular temperature gradient reported by Lee et al. [16,29]. This gradient was validated against 18F-FDG-PET/CT and infrared thermography in 87 lean individuals [29] while the validity of other gradients has not yet been proved [12,17].

Interestingly, we identified the right supraclavicular skin temperature as the only marker that did not decrease during cold exposure (Figure 6), which is in line with the hypothesis that cold exposure activates BAT, and that BAT generates heat. Besides, this region was properly covered by the cooling vest. Although we have no data on BAT activity and volume of the participants, this finding concurs with other studies that showed that supraclavicular skin temperature was positively associated with BAT activity and volume in the supraclavicular zone in healthy young men [12,14,15,17,45]. However, we cannot ignore that the absence of supraclavicular skin temperature decrease upon cold exposure is due to the presence of large blood vessels (i.e. aorta) close to the skin in this area. More studies are required to confirm this finding, as well as to elucidate the role of body mass index and subcutaneous adipose tissue in the measurement of all parameters of skin temperature [45–48]. Peripheral temperature gradient is a proxy of peripheral vasoconstriction. This gradient has been used as a marker for changes in the blood flow in peripheral zones. The peripheral vasoconstriction is a strategy of the body to keep the organs warm during cold exposure [5] carrying some of the peripheral blood to the central part of the body. House & Tipton [37] proposed a gradient between the temperature of the right top of the forefinger and the temperature of the middle part of the right forearm. They validated this gradient against laser Doppler flowmetry and reported that a difference of 2°C may indicate peripheral vasoconstriction. In contrast, a gradient lower than 0°C suggests peripheral vasodilatation. Sessler et al. [24,49] reported vasoconstriction by a difference of ≥4°C, while a difference lower than 4°C points to peripheral vasodilatation. Of note is that both thresholds are reached in our experimental conditions. However, other studies used the same peripheral temperature gradient as a proxy of peripheral vasoconstriction but in different anatomical positions [14] or calculated the gradient in a different way [25]. The best way to estimate peripheral vasoconstriction is currently not known and further studies are warranted. We calculated the same gradient but in the lower part of the body (instep-gastrocnemius) and observed an increase of this gradient after cold exposure. Therefore, a peripheral vasoconstriction occurs in the lower part of the body, as it happens in the upper part of the body, as previously reported [14,50].

Limitations

Results of this study should be considered with caution. Data are based on a single cooling protocol, using a cooling vest (that covers only chest and back zones), and we do not know whether the results apply to different cooling protocols or instruments (e.g. cooling blankets or ice blocks) or to longer cold exposure after shivering occurs. Additionally, the study was conducted on young lean men and we do not know whether these results apply to older people, to women, or to persons with higher (or lower) levels of body fat or in narrower ranges of hours. This study was performed in the south of Spain, and the equations were used under a personalised cooling protocol. Therefore, we are unaware if the results match those of other countries or environments and whether they can differ under warming or exercise protocols. We cannot ignore that the time of the day when our study was conducted may have influenced the results. Due to the methodological nature of this study, our findings are not comparable with other studies because they have used other cooling or warming protocols [7,14,18–23].

CONCLUSION AND RECOMMENDATIONS

We detected differences in skin temperature across the studied equations in both warm and cold room conditions. Based on these findings, we suggest a set of 19 ibuttons to estimate mean, proximal, and distal skin temperatures as well as body temperature gradients. We recommend to measure mean skin temperature with the 14-ISO equation [41]; proximal and body gradient of skin temperature with the Boon et al. equation [15]; distal skin temperature with the Krauchi et al. equation [36]; supraclavicular temperature gradient with Lee et al. equation [16,29] and peripheral temperature with Sessler et al. equation [3] (Table 3). These equations are based on the 14 anatomical positions reported by ISO STANDARD 9886:2004 [41] plus five more ibuttons placed on the right supraclavicular fossa, right middle clavicular bone, right middle upper forearm, right top of forefinger, and right upper chest (Figure 1: ibuttons 1-16, 20, 21, and 25, respectively). Moreover, we have seen that all selected equations are sensitive to the cooling protocol study (see Figure 6), except the supraclavicular skin temperature which was similar across temperature conditions.

Table 3. Recommended equations to measure skin temperature.

Outcome	Reference	ibuttons (n)	Anatomical positions (Fig. 1)	Equation	Rationale to select the equation
Mean skin temperature	14 ISO 9886-2004 [41] (14-ISO)	14	From 1 to 14	(Forehead*0.07) + (Neck*0.07)+ (Right Scapula*0.07) + (Left Chest*0.07) + (Right Deltoid*0.07) + (Left Elbow*0.07) + (Right Abdomen*0.07) + (Left Hand*0.07) + (Left Lumbar *0.07) + (Right Thigh*0.07) + (Left Hamstring*0.07) + Right Shinbone*0.07) + (Left Gastrocnemius*0.07) + (Right Instep*0.07)	Mean skin temperature estimated with 14-ISO was similar to that estimated using 26 ibuttons (PROFITH equation), which suggests that the 14-ISO equation covers the most important body sites with less ibuttons.
Proximal skin temperature	Boon et al. [15] (Boon)	3	10,16,8	(Right Thigh*0.383) + (Right Clavicular*0.293) + (Right Abdomen*0.324)	This equation includes less ibuttons than other equations, and it also includes an ibutton at the clavicular site which is close to BAT deposits.
Distal skin temperature	Kräuchi et al. [36] (Krauchi)	2	9, 14	(Left Hand+Right Instep)/2	Distal skin temperature estimated with this equation was similar to that estimated using 6 ibuttons (PROFITH equation), which suggests that this equation covers the most important body sites with less ibuttons.
Body temperature gradient	Boon et al. [15] (Boon)	5	9,14,10,16,8	[(Left Hand+Right Instep)/2] - [(Right Thigh*0.383) + (Right Clavicular*0.293) + (Right Abdomen*0.324)]	Body temperature gradient estimated with this equation was similar to that estimated using 11 ibuttons (PROFITH equation), which suggests that this equation covers the most important body sites with less ibuttons.
Supraclavicular temperature gradient	Lee et al. [29] (Lee S-RC)	2	15, 25	(Right Supraclavicular(S) - Right Chest (RC))	This equation has been validated against 18F-FDG-PET/CT and infrared thermography.
Peripheral temperature Gradient	Sessler et al. [3] Right arm	2	20, 21	(Right Forearm-Right Top of forefinger)	This equation has been validated against laser Doppler flowmetry.

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A methodological approach to improve skin temperature measurement using iButtons in human cold-induced studies

CHAPTER 4

BACKGROUND

In 1937, Hardy and Du Bois studied the effect of cold vs. warm exposure on the temperature of the skin [1]. Since then, several studies have included skin temperature measurements in response to different stimuli, such as anesthetics [2], food [3], heat [4] and cold [5] environment in order to quantify their thermoregulatory responses. Moreover, some studies are able to estimate indirect markers of human circadian rhythmicity based on wrist temperature (4,5).

Such thermoregulatory responses are commonly studied with a set of different devices attached to the skin [5]. iButtons are disc-shaped sensors of small dimensions (16x6 mm2) and low cost (approx. USD50), with a stable and autonomous system that measures temperature and records the data in a protected memory section [8]. The manufacturer's instructions explain that iButtons are composed of a small battery and temperature sensor placed closely behind the part of the side at which the identification number has been carved. It was previously reported that iButtons are able to measure at both sides [8,9], with the side opposite to the thermometer sensor yielding the most accurate [8] and faster response to thermal changes [9].

Since the discovery that human adults have metabolic active brown adipose tissue (BAT) that can be activated by cold exposure [10,11], studying the human thermoregulatory responses to cold stimuli has acquired much attention [12] and iButtons are the devices most used in these experiments. Nevertheless, the fact that iButtons are measuring temperature by both sides [8,9] has gone unnoticed and how this "detail" affects the skin temperature measurement has not been addressed.

Therefore, the aims of the present study are: (i) to confirm that iButtons measure at both sides, and (ii) to find and validate a solution for avoiding the temperature measurement of iButtons at both sides.

MATERIAL AND METHODS

Three experiments were conducted: for the first and the second experiment, we used 7 iButtons, whereas for the third experiment we used 34 iButtons (DS-1922 L, - Thermochron; Maxim, Dallas, USA). In the first and second experiment, the temperature was recorded at 1-second intervals during 30 minutes, whereas in the third experiment the skin temperature was recorded at 1-minute interval during 180 minutes. The configuration of the iButtons was at their highest resolution (0.0625°C). In the third experiment, we calculated mean, proximal and distal skin temperature [3,5]. All data recorded by the devices and equations were analyzed by Temperatus software (http://profith.ugr.es/temperatus).

First experiment

We used two thermal packs, of which one was cooled down in a fridge (4°C) and the other one was heated in the microwave just before starting the measurement. Then, we placed two iButtons adjacent on a table in opposite positions. We covered these iButtons with the warm thermal pack and placed two additional iButtons on top, again in opposite positions. These iButtons were then covered with the cold thermal pack and two additional iButtons were placed over the cold thermal pack. Another iButton was used to record the ambient temperature (Figure 1A).

Second experiment

Foam polyethylene is a cheap and insulating material, which is often used to insulate pipelines exposed to extreme temperatures. We attached small pieces of polyethylene (dimensions: 4 cm x 4 cm x 0.8 cm; length x width x height) to iButtons: two iButtons were isolated from one side and other two from the other side (Figure 2A). We put a thermal pack in a freezer at -20°C during 48 hours before performing the experiment. Before the experiment started, we placed two iButtons in opposite positions on a piece polyethylene on the table; two iButtons in opposite positions on a table and covered with a piece of polyethylene; and two iButtons free of polyethylene as controls (on the table, between the isolated iButtons). On top of the 6 iButtons, we placed the frozen

thermal pack. Another iButton was used to record the ambient temperature (Figure 2B).

Third experiment

One healthy female volunteer (23 years old, body mass index: 21.6 kg/m^2), who refrained from any type of exercise 48 hours before the experiment, came to the lab in a 6 hours-fasting state. We attached the iButtons covered with polyethylene and the non-isolated iButtons adjacently at 17 skin areas as described elsewhere (2 iButtons/skin area) [3]. All iButtons faced the skin by the side opposite to the identification number. With all iButtons attached, she was acclimated in a warm room (23.8°C) during 30 minutes and subsequently she was transferred to another room (22.8±0.4°C). After 15 minutes, she was dressed up with a water-perfused cooling vest (Polar Products Inc., Ohio, USA) (Figure 3A). The total record of skin temperature was 180 minutes (last 10 minutes in the warm room + 170 minutes in the cold room). We progressively decreased the water temperature of the cooling vest until a minimum temperature of 3.9°C was reached in approx. 155 min (Figure 3B). A detailed description of the cooling protocol can be found elsewhere [13].

RESULTS

First experiment

Figure 1A graphically shows the design of the experiment. This experiment shows that iButtons are measuring by both sides (Figure 1B). iButtons number (n°) 2, 4 and 6 were placed with the identification number to the places (table, warm or cold thermal packs), whereas n° 1, 3 and 5 were placed, next to 2, 4 and 6, but by the other side. Figure 1B shows the measurement of iButtons every second during 30 minutes. Dash lines represent the iButtons that were placed with the identification number to the stimuli, whereas continuous lines represent the iButtons that were placed by the other side. Figure 1B also shows that the more accurate measurements were performed by the iButtons that were placed by the side opposite to the identification number. iButtons n° 3 and 4 show that these devices are measuring by both sides. Moreover, we observed the largest difference between the temperature measured by iButtons placed in the same location but in different positions in the cold stimulus (Figure 1C).

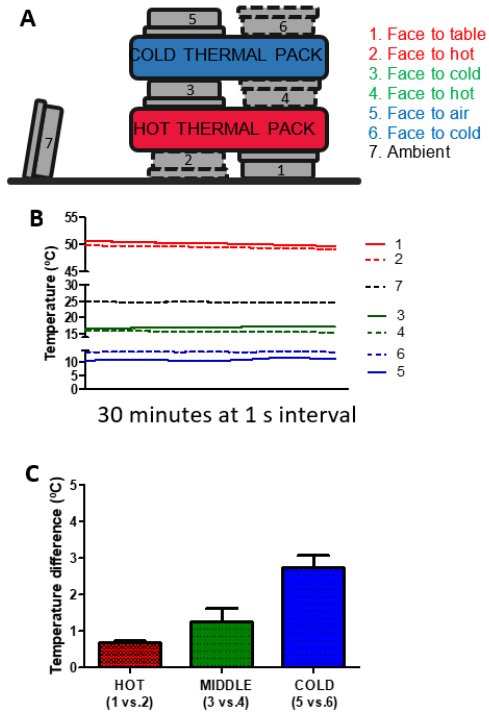

Figure 1. iButtons are registering temperature by both sides. A. Experimental design to demonstrate the problem with iButtons. B. Kinetics of the measurements of iButtons during 30 minutes at 1 s intervals. C. The average difference in temperature between iButtons in different stimuli.

Second experiment

This experiment shows that isolated iButtons performed a more accurate measurement of the temperature of the frozen thermal pack. Figure 2A shows the design of the experiment. iButton n° 1 was faced by the identification number to the frozen thermal pack with a piece of polyethylene attached by the other side, whereas n° 2 was placed next to n° 1 but the polyethylene was placed in the other side. iButton n° 6 was faced by the identification number to the table, but a piece of polyethylene was placed between

the iButton and the frozen plate. iButton n° 5 was placed next to n° 6 by the opposite side. iButtons n° 3 and 4 were placed on the table with no insulation, as controls. Figure 2C shows that iButtons n° 1 and 2 measured a colder temperature in comparison to the rest of iButtons, when the devices were isolated from the table. We found that iButtons n° 5 and 6 measured coldest temperature of the table, when they were isolated of the frozen thermal pack, in comparison to the non-isolated iButtons n° 3 and 4. The non-isolated iButtons measured higher temperatures in comparison to n° 5 and 6, because they were in contact with the table and the air (20°C), which was slightly hot in comparison to the frozen thermal pack.

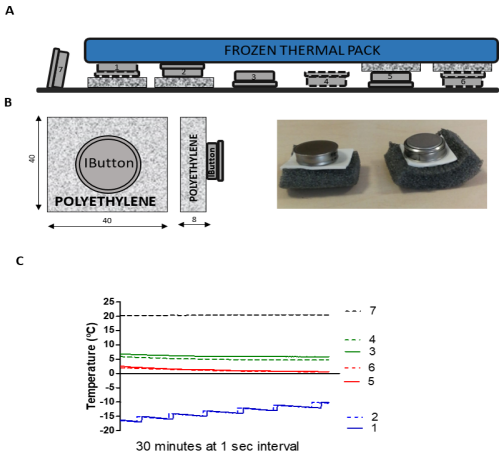

Figure 2. Solution to avoid that the iButton registers temperature for both sides in cold conditions. A. iButton covered by a short piece of polyethylene. B. Experimental design to define how the polyethylene works in these devices. The measurements are reported in milimiters. C. Kinetics of the measurements of iButtons during 30 minutes at 1 s intervals.

Third experiment

This experiment showed that the iButtons isolated with polyethylene systematically measured higher, mean, proximal, distal, supraclavicular, chest and fingertip skin temperatures (Figure 3C, D, E, F, G and H) at all ambient temperatures, in comparison to those iButtons that were not isolated (Figure 4). These differences were around 1-2 °C.

DISCUSSION

This study confirms that iButtons are measuring by both sides and shows that the flat part of the iButtons (opposite to the identification number) performed more accurate measurements of temperature. We additionally show that covering the iButton with polyethylene is a valid and cheap solution to isolate one side of the device. Future studies using iButtons in human physiology experiments should isolate the devices in order to obtain accurate and valid skin temperature data. The results of those studies that did not isolate iButtons should be interpreted with even more caution.

The device manufacturer does not specify which side of the data logger should be placed on the surface of interest to obtain a temperature measurement [9]. Hasselberg et al. [9] performed a small experiment with 12 iButtons and concluded that the top side or ceiling of the iButton had a faster response to thermal changes, which may have been caused by the location of the battery (toward the ceiling) and the temperature sensor (toward the base) of the data logger. The thermometer inside the iButtons is mainly composed by plastic (which is not a good temperature conductor), whereas the battery is composed by a set of materials that conducts quite well the temperature.

Therefore, the explanation about why iButtons are measuring more accurately the temperature for the opposite side where the thermometer is placed, could be that the battery is able to keep the temperature inside the case, being in direct contact with the thermometer sensor. Our proposal of isolated iButtons with polyethylene seems to be a valid, relatively easy and inexpensive solution to correct the detected measurement error. Surprisingly, we observed that even in warm conditions, when the participants were not dressed with the cooling vest, all parameters of skin temperature had higher values, which means that all studies that have measured skin temperature in a room in response to exercise, to a meal test or in chronobiological studies should introduce isolated iButtons.

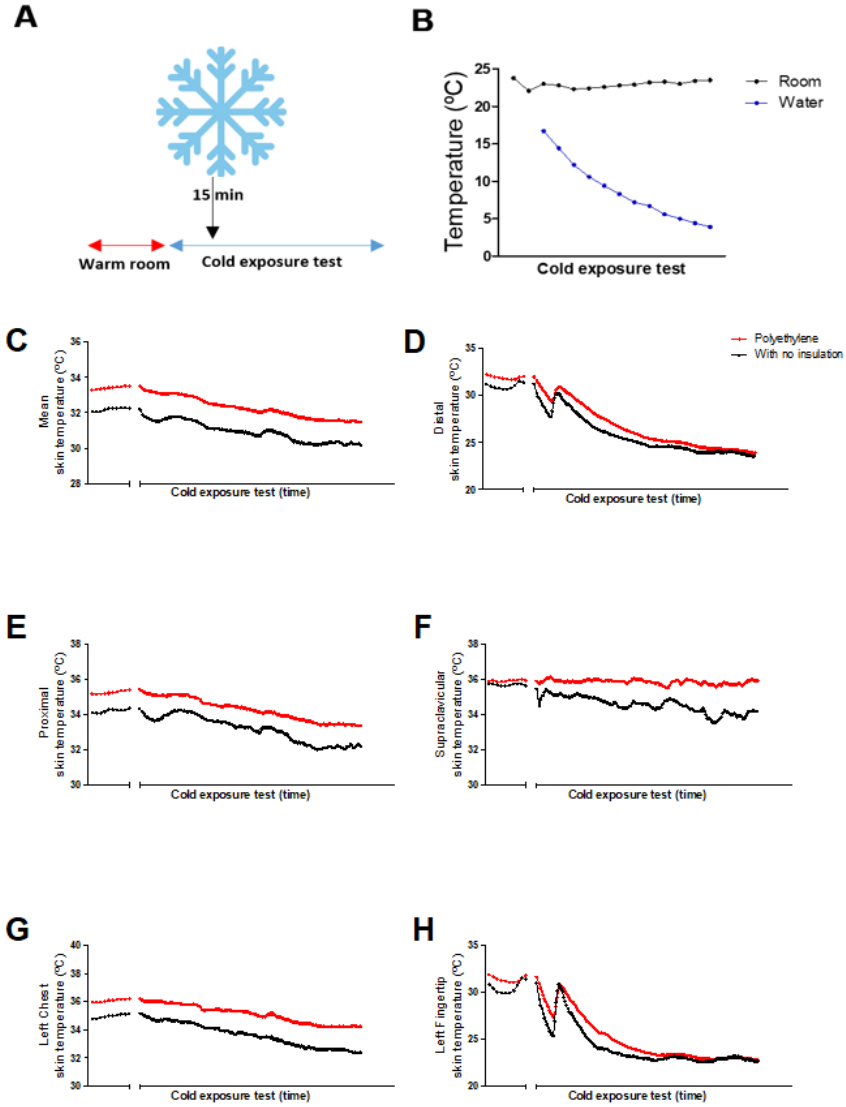

Figure 3. Temperature data record during a cooling down protocol. Data measured in one participant. Red lines refer to iButtons with polyethylene, whereas black lines refer to iButtons with no isolation.

Moreover, we found that the supraclavicular skin temperature isolated by polyethylene was kept constant during the cooling protocol, whereas when the device that was not isolated it slightly decreased after the cold exposure, most likely due to the contact with the vest. This finding has implications for the validity of supraclavicular skin temperature as a proxy of 18F-Fluorodeoxyglucose uptake by BAT, as previously reported [14,15]. In addition, we believed that the iButtons with polyethylene, which were placed in areas not covered by the cooling vest, would obtain similar values to the non-isolated iButtons (i.e. distal skin temperature or left fingertip, Figure 3D and E). However, we found differences, similar to those observed in the iButtons that were in

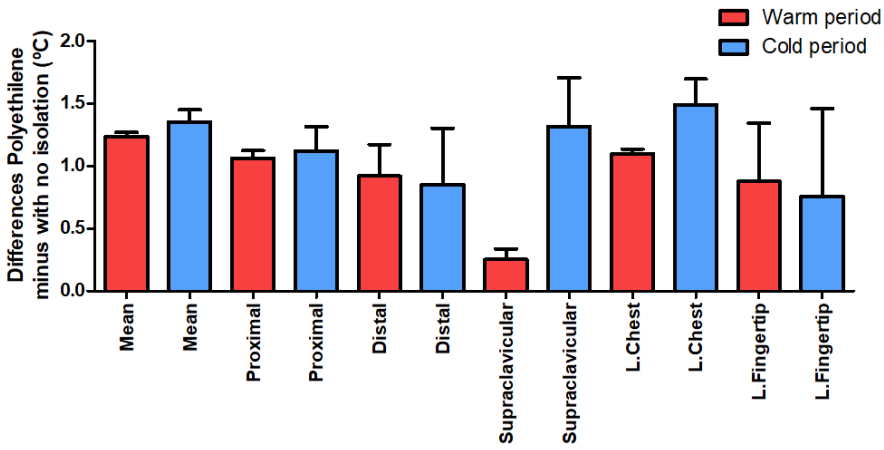

Figure 4. Each bar represents the difference between the mean value of the iButton covered by polyethylene and with no isolation during warm period (red bars) and cold period (blue bars). These graphs represent mean, proximal, distal, supraclavicular, left chest and left fingertip of 1 female individual. Values are presented as mean and standard deviation. All the results are statistically different from 0 (all $P<0.001$).

contact with the cooling vest. This finding means that independently of whether the iButtons were covered or not by the cooling vest, the devices that were not isolated systematically measured lower temperatures.

CONCLUSIONS

iButtons are registering temperature by both sides, and the output seems to be an average of both sides, showing that the flat side showed more accurate measurements. We propose and validate an isolated alternative based on polyethylene. With this isolation we obtain more accurate measurements of skin temperature in both warm and cold conditions.

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PART 2

The importance of understanding the instruments: brown adipose tissue methodology

A new personalized cooling protocol to activate brown adipose tissue in young adults

CHAPTER 5

BACKGROUND

Obesity prevalence has increased exponentially during the last decades, and estimations indicate that global obesity prevalence will reach 18% in men and surpass 21% in women by 2025 [1]. Obesity is associated with a number of conditions and pathologies including insulin resistance, dyslipidaemia, type 2 diabetes, and cardiovascular diseases [2].

Brown adipose tissue (BAT) has the ability to oxidise glucose and lipids and to dissipate energy as heat which makes it an attractive target for anti-obesity and related comorbidities [3]. BAT is mainly regulated by the sympathetic nervous system to defend core body temperature when mammals are exposed to temperatures below thermoneutrality [3–5]. In 2009, a number of human studies showed that BAT is both present and thermogenically active in adults [6–9].

To date, the gold standard to quantify human BAT volume and activity is 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography-computed tomography (PET/CT) [10]. Participants should be exposed to cold for a minimum of ~60 minutes prior to the injection of 18F-FDG and for ~60 minutes after the injection to maximize BAT activity and thus 18F-FDG uptake by the tissue [11]. However, studies have used different cooling protocols to activate human BAT [11,12]. Whereas some used cold exposure to a fixed and predefined temperature for all participants [8], others used personalised cooling protocols, where the temperature is adjusted to the individual's shivering threshold. A fixed protocol could induce sub-maximal non shivering thermogenesis, whereas the personalised cooling protocol is likely to induce maximal non shivering thermogenesis. Moreover, several authors applied different methodologies to induce cold stimuli including ice-blocks [17], cooling vests [18], cooling blankets [15] and air conditioning [19], among others [12], in combination with fixed or personalised cooling protocols. Therefore, the effect of these multiple combinations on the activation of human BAT is unknown.

Recently, an expert panel recommended the use of personalised cooling protocols to quantify BAT in humans, especially after an intervention that is expected to change BAT volume or activity [11]. Brown Adipose Reporting Criteria in Imaging STudies (BARCIST 1.0) [11] recommendations include (i) determination of the shivering threshold and (ii) cold exposure at a relative shivering threshold temperature of the individual for 2 hours prior to the PET/CT scan [11] on the same day, as other authors have done previously. Therefore, the main difference between these studies and the present study, is that we measured the shivering threshold 48-72 hours before to perform the PET/CT scan, in order to avoid excessive cold stress during BAT measurements.

The aim of the present study was to determine the effect of a novel personalised cooling protocol where the shivering threshold was measured on a separate day, on BAT volume and activity in young adults.

MATERIAL AND METHODS

A total of 47 white Caucasian young adults (n=28 women) aged 22±2 years participated in the study (Table 1). Participants were enrolled in the ACTIBATE study [18], an exercise-based randomized controlled trial (ClinicalTrials.gov ID:NCT02365129). All participants were healthy, sedentary (<20 min physical activity on <3 days/week), non-smokers, had no family history of type 2 diabetes, and did not take any medication that could influence the cardiovascular or thermoregulatory responses to cold exposure. The study protocol and 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 (n°924) and of the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada). The evaluations were performed in four waves of ~12 participants each, from 15th October to 28th November, 2015 in Granada (Spain).

A

Room temperature (ºC)	WARM ROOM	22.1 ±1.6	COLD ROOM	19.8±0.5											
Water Temperature of the cooling vest (ºC)					16.6	14.4	12.5	10.5	9.4	8.3	7.2	6.6	5.5	5	4.4	3.8
Time (min)		30		15	10	10	10	10	10	10	10	10	10	15	15	45
Accumulated phase time (min)		30		15	25	35	45	55	65	75	85	95	105	120	135	180

B

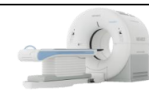
Room temperature (ºC)		22.2±0.5		20.2±0.3			PET/CT scan
Water temperature of the cooling vest (ºC)	WARM ROOM	Shivering achieved on STT	COLD ROOM	~4 above STT	Injection ^{18}F-FDG	+ ~1	
		Shivering not achieved on STT		3.8		+ ~1	
Time (min)		30		60		60	12

Figure 1. A. Shivering threshold test (STT) protocol. B. Personalised cooling protocol prior to 18F-fluorodeoxyglucose (18F-FDG)-Positron emission tomography/computed tomography (PET/CT) scan

Previous conditions to the study days

Participants arrived to the research centre by bus or by car, and in fasting conditions (at least 6 hours). They were advised to (i) sleep as usual, (ii) refrain from any moderate (for 24 hours) or vigorous (for 48 hours) physical activity, and (iii) to not consume alcoholic or stimulant beverages (for 6 hours), or drugs affecting peripheral circulation (24 previous hours). We encouraged them to drink room-temperature water before the tests, ~1 litre before the shivering threshold test (STT) and ~2 litres before the PET/CT scan.

Shivering threshold test

We conducted the STT 48-72 hours before applying 2 hours of personalised cold exposure prior to the PET/CT scan. Upon arrival to the research centre, all participants confirmed that they had followed the pre-study instructions. They emptied their bladders, dressed up with standardized clothes (sandals, T-shirt and shorts, clo-value: 0.20 [20]), and entered into a warm room (22.1±1.6°C) where they remained seated for 30 minutes (Figure 1A). Participants received detailed information and instructions about the STT protocol. Afterwards, they entered into a cold room (19.8±0.5°C) where they remained seated in a chair for 15 minutes, and were not allowed to stand up, move, rub or cover their bodies. Then, participants dressed up with a temperature-controlled water perfused cooling vest (Polar Products Inc., Ohio, USA), which covers the clavicular region, as well as the chest, abdominals, and the back. Water temperature was set at 16.6°C and decreased progressively every 10 minutes until 5.5°C (Figure 1A). If participants did not report shivering and researchers did not observe it either, we decreased water temperature by 0.6°C every 15 minutes until 3.8°C. At this stage, if shivering had not occurred, participants remained in the cold room for another 45 minutes, after which the test was finished (Figure 1A). Women were kindly asked to tie up their hair to reduce hair insulation over the neck and shoulders. We determined shivering visually and by asking the participants if they were experiencing shivering. We had previously observed that

both self-reported and visual inspection of shivering concurred with muscle activity measured by EMG in an independent group of 6 young adults (unpublished observations). We recorded whole-body, clavicular, and hands thermal sensation at the end of the warm period and at the end of the STT using a continuous 7-points thermal sensation interval scale (American Society of heating, refrigerating and air conditioning engineers, ASHRAE) [21,22]. Participants also reported the subjective perception of shivering in a numeric rate scale (NRS) where 0 refers to "I am not shivering" and 10 refers to "I am shivering a lot".

Personalised cooling protocol prior to PET/CT scan.

Participants confirmed that they had followed all pre-study conditions and were invited to empty their bladders and dressed up with the standardized clothes (the same as the STT day). They stayed in a warm room for 30 minutes (22.2±0.5°C), after which they entered into a cold room (20.2±0.3°C). As in the STT day, participants wore the same temperature controlled water perfused cooling vest (Polar Products Inc., Ohio, USA) for 60 minutes set at ~4°C above the temperature that caused the onset of shivering. If the participant did not report shivering in the STT, water temperature was settled at 3.8°C, similar to other personalised cooling protocols. Participants were instructed to immediately inform the researchers if they experienced shivering at any time. When shivering was reported, we increased the water temperature by 1°C and provided a bathrobe for 2 minutes until shivering disappeared. After 60 minutes of personalised cold exposure, we administrated an intravenous 18F-FDG injection (185 MBq; approx. 2.78 MBq/kg), and we increased water temperature by approx. 1°C. This temperature was kept constant for another 60 minutes (Figure 1B). After 2 hours of personalised cold exposure, participants went into the PET/CT scan (Siemens Biograph 16 PET/CT, Siemens, Germany). For the CT acquisition a peak kilovoltage of 120 was applied, while for the PET acquisition a scan time of 6 min per bed position was set. In total, 2 bed positions were scanned from atlas vertebrae to thoracic vertebrae 4. Participants reported their thermal sensation (by ASHRAE-scales) and the subjective level of shivering (NRS) in the warm room and at the end of the cooling exposure period in the same way as in the STT day.

PET/CT analysis

The PET/CT scans were analysed using Beth Israel plugin for FIJI [8] software (http://sourceforge.net/projects/bifijiplugins/ [23]). We calculated the standardized uptake value (SUV) as [18F-FDG uptake (kBq/mL)/(injected dose [kBq]/patient weight [g])]. SUV threshold was calculated as SUV≥ 1.2/(lean body mass/body mass) [11]. We applied a fixed range of Hounsfield units (HU, -190 to -10) [11]. The region of interest (ROI) was semi-automatically outlined from atlas vertebrae (Cervical 1) to thoracic vertebrae 4.

We determined BAT volume, SUVmean, BAT metabolic activity, SUVpeak, and SUVmax according to BARCIST 1.0 recommendations [11,24]. Furthermore, we also considered as BAT-depots all pixels that achieved the predefined thresholds of SUV and HU. [11]. The PET/CT scans were visually and carefully examined to detect 18F-FDG uptake in BAT-specific depots. Participants were categorized as PET+ when BAT volume was ≥5 ml and 18F-FDG uptake was clearly apparent, and as PET- when BAT volume was <5 ml and there were no signs of cold-stimulated 18F-FDG uptake in the BAT region [25]. Body composition was measured on a separate day by Dual Energy X-ray Absorptiometry (HOLOGIC, QDR 4500W) [18].

Statistical analysis

Data are presented as mean ±standard deviation, unless otherwise stated. We used a one-way analysis of variance (ANOVA) to test differences in body composition and in BAT outcomes by cold-stimulated 18F-FDG uptake by BAT (PET+ vs. PET-) and by sex (men vs. women). Categorical variables (sex and weight status) were compared using the X^2 test. We used a paired t-test to study

differences on self-reported thermal sensations and shivering between the STT and the personalised cooling protocol prior to the PET/CT scan in both warm period and at the end of the cooling exposure. We estimated the effect size as previously reported [26]. We established as dependent variable BAT-related outcomes and as independent variable sex. We found a medium effect for BAT volume (d=0.45), a moderate effect for SUVpeak (d=0.63) and SUVmax (d=0.69) and a large effect for SUVmean (d=1.08). Moreover, when we established as independent variable PET + or PET- groups the effect size of BAT-related outcomes increased even more (all d≥2.07). Analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM SPSS Statistics, IBM Corporation) and the level of significance was set to <0.05.

RESULTS

Characteristics of the participants

There were no differences of age or fat mass between sexes (P>0.2), yet men had a higher BMI and lean mass than women (all P≤0.001) (Table 1). However, we did not find significant differences in fasting glucose and insulin levels between sexes (P=0.243 and P=0.569, respectively).

Table 1. Characteristics of participants by sex.

	Men (n=19)			Women (n=28)			P
Age (years)	22.1	±	2.1	21.9	±	1.8	0.760
BMI (kg/m2)	27.6	±	5.2	23.2	±	3.6	**0.001**
Fat mass (kg)	25.9	±	9.9	23.2	±	7.9	0.294
Lean mass (kg)	53.4	±	7.7	35.0	±	5.1	**<0.001**
Fasting glucose (mmol/l)	4.9	±	0.4	9.3	±	6.3	0.243
Fasting insulin (µIU/ml)	4.8	±	0.4	8.3	±	4.8	0.569

Data are presented as mean and standard deviation. BMI: Body mass index. P for sex comparisons.

Cooling protocol

A total of 38 participants (n=25 women) reached shivering during the STT and 9 participants (n=3 women) did not. There were no significant differences in BAT-related outcomes between both groups (all P>0.3, data not shown). There were no significant sex differences in mean water vest temperature at the end of STT (5.8±2.2 vs. 6.4±1.8 °C, men and women, respectively, P=0.875) or time until the end of STT (119.7±32.1 vs. 108.4±31.9 minutes, men and women, respectively, P=0.716).

Data on self-reported thermal sensation was available in 35 participants. The self-reported thermal sensation (assessed by ASHRAE scales) in the clavicular and hands zones as well as the subjective perception of shivering (assessed by NRS) were similar at the end of the warm period in both the STT and in the personalised cooling protocol before the 18F-FDG-PET/CT scan. However, whole-body thermal sensation was slightly higher (i.e.

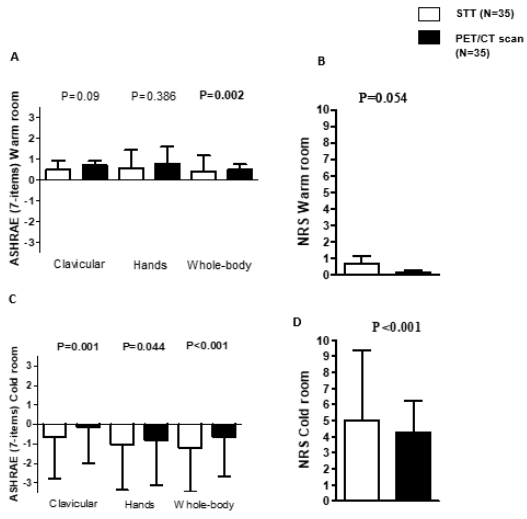


Figure 2. Self-report thermal sensation (in the clavicular, hands, and whole-body zones) and subjective perception of shivering in the shivering threshold test (STT) and in the personalised cooling protocol before 18F-fluorodeoxyglucose (18F-FDG)-Positron emission tomography/computed tomography (PET/CT) scan. A. American Society of heating, refrigerating and air conditioning engineers (ASHRAE) scales of 7 points: -3=cold, 0=neutral, and 3=hot in the warm room. B. Numeric rate scale (NRS): 0= "I not shivering" and 10= "I am shivering a lot" in the warm room. C. ASHRAE scales of 7 points in the cold room. D. NRS in the cold room. All data are represented as mean and standard deviation. P for STT vs. PET/CT comparisons.

warmer, P=0.002) at the end of the warm period on the STT day (Figure 2A). The self-reported thermal sensation and the subjective perception of shivering were significantly higher at the end of the STT than in the personalised cooling protocol before the 18F-FDG-PET/CT scan (i.e. cooler, all P≤0.05, see Figure 2C and D).

warm room. B. Numeric rate scale (NRS): 0= "I not shivering" and 10= "I am shivering a lot" in the warm room. C. ASHRAE scales of 7 points in the cold room. D. NRS in the cold room. All data are represented as mean and standard deviation. P for STT vs. PET/CT comparisons.

Table 2. Characteristics of participants by positron emission tomography with positive glucose uptake (PET+) vs. negative glucose uptake (PET-).

	PET+ (n=40)	PET- (n=7)	P
Age (years)	21.8 ± 1.9	23.1 ± 1.3	0.086
Sex (n,%)			0.329
Men	15 (38)	4 (57)	
Women	25 (62)	3 (43)	
BMI (kg/cm2)	25.3 ± 5.0	23.2 ± 2.6	0.297
Weight status (n,%)			0.276
Normal-weight	22 (55)	6 (85.7)	
Overweight	11 (27.5)	1 (14.2)	
Obese	7 (17.5)	0	
Fat mass (kg)	25.2 ± 8.8	19.3 ± 6.6	0.101
Fat mass index (kg/cm2)	9.0 ± 3.0	6.7 ± 2.9	0.072
Lean mass (kg)	41.9 ± 11.3	45.3 ± 9.0	0.457
Lean mass index (kg/cm2)	14.9 ± 3.3	15.4 ± 2.2	0.727
SUV threshold (g/ml)	2.1 ± 0.2	1.9 ± 0.3	**0.035**
BAT volume (ml)	96 ± 58	1.5 ± 1.7	**<0.001**
Metabolic activity (g)	467 ± 344	3.3 ± 3.6	**0.001**
SUVmean (g/ml)	4.5 ± 1.5	1.9 ± 0.9	**<0.001**
SUVpeak (g/ml)	11.8 ± 6.1	2.7 ± 1.4	**<0.001**
SUVmax (g/ml)	14.9 ± 7.6	2.9 ± 1.5	**<0.001**

Data are presented as mean and standard deviation, unless otherwise stated. P for PET+ vs. PET- comparisons. BAT: Brown adipose tissue; BMI: Body mass index; SUV: Standardized uptake value.

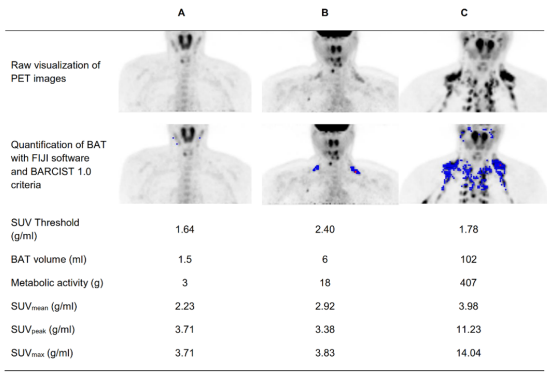
Figure 2. Self-report thermal sensation (in the clavicular, hands, and whole-body zones) and subjective perception of shivering in the shivering threshold test (STT) and in the personalised cooling protocol before 18F-fluorodeoxyglucose (18F-FDG)-Positron emission tomography/computed tomography (PET/CT) scan. A. American Society of heating, refrigerating and air conditioning engineers (ASHRAE) scales of 7 points: -3=cold, 0=neutral, and 3=hot in the

18F-FDG-PET/CT measurements

We identified 40 participants (25 women) as PET+ and 7 (3 women) as PET-. Figure 3 shows an example of a PET- participant with negligible cold-stimulated BAT 18F-FDG uptake (Figure 3A), a PET+ participant with the low cold-stimulated BAT 18F-FDG uptake (Figure 3B), and a PET+ participant with high cold-stimulated BAT 18F-FDG uptake (Figure 3C). Mean water vest temperature at the end

of STT and time up to the end of STT was similar in the PET+ and PET- groups (6.1±1.9 vs. 6.7±2.1 °C, respectively, P=0.994; and 115.2±30.1 vs. 100.3±42.5 minutes, respectively, P=0.291). There were no significant differences on self-reported thermal sensation between PET+ and PET- group (all P>0.1, data not shown).

Table 2 shows the participants' characteristics categorised as PET+ and PET-. There were no statistical significant differences with respect to age, sex, or body composition between groups (all P>0.05) (Table 2). The PET+ group had significantly higher SUV threshold (P=0.035), BAT volume (P<0.001), BAT metabolic activity (P<0.001), SUVmean (P<0.001), SUVpeak (P<0.001), and SUVmax (P<0.001) than their PET- counterparts (Table 2).



	A	B	C
SUV Threshold (g/ml)	1.64	2.40	1.78
BAT volume (ml)	1.5	6	102
Metabolic activity (g)	3	18	407
SUV_{mean} (g/ml)	2.23	2.92	3.98
SUV_{peak} (g/ml)	3.71	3.38	11.23
SUV_{max} (g/ml)	3.71	3.83	14.04

Figure 3. Visual determination of a positron emission tomography with a participant positive for 18F-FDG uptake (PET+) and a participant negative for 18F-FDG uptake (PET-). A. A PET- participant with negligible cold-stimulated brown adipose tissue (BAT) 18F-FDG uptake. B. A PET+ participant with the lowest cold-stimulated BAT 18F-FDG uptake. C. A PET+ participant with high cold-stimulated BAT 18F-FDG uptake. BMI: Body mass index; SUV: Standardized uptake value.

Within the PET+ group, SUV threshold was higher in women than in men (2.1±0.2 vs. 1.9±0.2 g/ml, respectively, P=0.003, Figure 4A). Women also presented higher SUVmean (5.0±1.6 vs. 3.6±0.9 g/ml; P=0.003, Figure 4D), slightly higher SUVpeak (13.2±6.4 vs. 9.6±5.0 g/ml; P=0.068, Figure 4E), and higher SUVmax (16.7±7.9 vs. 11.9±6.0 g/ml; P=0.05, Figure 4F) than men. There were no sex differences in BAT volume (86±53 ml vs. 113±65 ml, women and men, respectively, P=0.161, Figure 4B) or BAT metabolic activity (478±361 vs. 448±325 g, respectively, P=0.797, Figure 4C).

DISCUSSION

In the present study we describe a new personalised cooling protocol in young adults that follows the BARCIST 1.0 recommendations to determine BAT volume and activity [11] where the shivering threshold was determined on a separate day. A total of 40 out of 47 (87%) participants were identified as PET+, and seven participants were identified as PET- even after 2 hours of personalised cold-exposure prior to the PET/CT scan. As expected, PET+ participants had higher BAT volume, BAT metabolic activity, SUVmean, SUVpeak and SUVmax than PET-. Of note, SUV threshold was higher in the PET+ group due to the fact that this group presented slightly higher levels of fat mass. PET+ women had higher BAT activity than PET+ men, whereas there were no sex differences in BAT volume.

To our knowledge, this is the first study that applied a 2-hour personalised cooling protocol prior to the PET/CT scan while determining the shivering threshold on a separate day (48-72 hours before). Of note is that all the personalised cooling protocols conducted the STT and the 2-hour cold exposure prior to the PET/CT scan on the same day. This design could be a burden as well as an extra cold-stress for participants, such as older [36] or unhealthy [37] participants. Moreover, personalised cooling protocols with shorter cold exposures could be useful to study the effect of certain drugs or in other population such as children, older people or unhealthy, to activate BAT. Retrospective studies showed that without prior cooling, BAT depots could be detected by PET/CT scan in ~6% of adults [38]. Nevertheless, when cooling exposure is applied prior to the PET/CT scan, the prevalence of PET+ is 20-31% in obese [30] and 40-100% in lean healthy adults. In the present study, the prevalence of PET+ in young adults was 85%, which concurs with previous observations. More studies are needed to elucidate which cooling protocol is more

efficient in terms of BAT activation and that at the same time causes participants less discomfort.

Recently, an expert panel launched a set of recommendations for conducting 18F-FDG-PET/CT experiments of human BAT, data analysis, and publication of results [11]. They suggested the use of a fixed criteria of HU (-190,-10) and an individualized SUV threshold based on body composition. They recommended the use of a SUV threshold of ≥1.2 divided by the participant's lean body mass/body mass [11] because 18F-FDG uptake is higher for lean tissue than for white fat. Therefore, women are expected to have a higher SUV threshold than men because they have lower levels of lean body mass and higher levels of white fat, which was confirmed in our study (Figure 4A). However, studies that used personalised cooling protocols did not find differences in BAT volume and activity between sexes [41], whereas in studies in which a fixed cooling protocol was used, women had higher activity than men [8]. These discrepancies could be based on different methodological protocols for BAT quantification [16]. Nevertheless, we applied a personalised cooling protocol and we found that women had higher activity than men. This finding could be based on the fact that women were leaner than men (see Table 1). Nevertheless, more studies are warranted to elucidate actual sex differences.

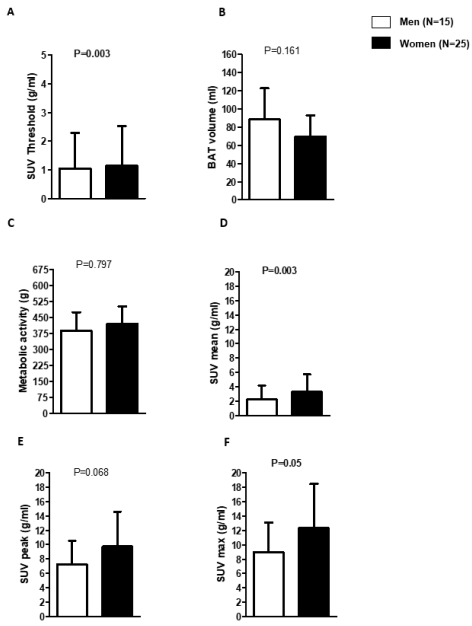


Figure 4. Brown adipose tissue-related outcomes in positron emission tomography in participants positive for 18F-FDG uptake (PET+) classified in men vs. women. Data are mean and standard deviation. P for sex comparisons. BAT: Brown adipose tissue; BMI: Body mass index; SUV: Standardized uptake value.

The levels of SUVmax observed in our study are in agreement with those reported by Bakker et al. [15] in Caucasians and south Asians (SUVmax: 15 g/ml), and by Hanssen et al. [42] in lean participants (SUVmax: 15.9±5.8 g/ml). Of note is that both studies applied a personalised cooling protocol. Taken together, these findings suggest that applying just 2 hours of personalised cold exposure prior to the PET/CT scan induces similar BAT activation in young adults than when both shivering threshold and 2 hours of cold exposure prior to the PET/CT scan are conducted on the same day. Levels of SUVmax might be less influenced by the methods used to quantify BAT, and it therefore allows between study comparisons. However, other BAT-related outcomes cannot be directly compared across studies without assuming that differences might be largely explained by the methodology used to quantify or activate BAT [16]. Therefore, the present study showed that a short cold exposure can induce BAT activation to a similar extent as longer cold exposures.

The observed subjective thermal sensations observed in our study are similar to other studies that used the same [43,44] or different instruments (e.g. visual analogue scales) [42,44–46]. As expected, participants had a higher thermal sensation and higher levels of subjective shivering during the STT, because the aim of this test was to produce shivering, while the aim of the personalised cooling protocol prior to the 18F-FDG-PET/CT scan was to produce maximal non shivering thermogenesis.

Limitations

The results of this study should be considered with caution. We performed the PET/CT scan from cerebellum to thoracic vertebrae 4, and

we may have therefore missed BAT depots localised in other areas. Nevertheless, most of the BAT depots detected in humans are localised in the areas covered by our scan [10,47]. The study was conducted in young men and women, and we do not know whether these results apply to older adults. It would be interesting to compare the results on BAT parameters as well as skin and core temperature in humans applying different cooling protocols, to validate if different cold exposures are able to activate human BAT. However, this is not possible due to the high radiation associated to the 18F-FDG-PET/CT scans. Furthermore, future studies should analyse the relationship between the BAT volume and activity and time to shivering. Finally, given the limited sample size of this study, which is therefore prone to type 2 errors, additional studies are needed to better understand the differences between PET+ and PET- groups.

CONCLUSION

Our cooling protocol is able to activate BAT in young adults. The novelty of our protocol resides in the fact that we applied just 2 hours of personalised cold exposure prior to PET/CT scan while the shivering threshold was determined on a separate day (48-72 hours before).

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The impact of using BARCIST 1.0 criteria on quantification of brown adipose tissue volume and activity in three independent cohorts of adults

CHAPTER 6

BACKGROUND

Brown adipose tissue (BAT) is present and metabolically active in human adults [1]. In 2009, several studies using 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET) combined with X-ray computed tomography (CT) (18F-FDG-PET/CT) imaging showed that human BAT can be activated upon cold exposure, that 18F-FDG uptake by BAT is more common in women than in men, and that 18F-FDG uptake by BAT is higher in lean than in obese individuals [2–5]. The capacity of BAT to combust energy as well as its beneficial role in glucose [6] and lipid [7] metabolism makes BAT an attractive therapeutic target in combating adiposity and type 2 diabetes.

Currently, 18F-FDG-PET/CT analysis is the most commonly used method to quantify human BAT volume and activity [1]. 18F-FDG-PET provides information about glucose uptake by metabolically active tissues including BAT, expressed as standardized uptake value (SUV)[8,9]. CT, on the other hand, provides anatomical information and allows the identification of various tissues, including adipose tissue and soft tissues, based on the radio-density expressed as Hounsfield units (HU). Therefore, quantification of BAT volume and activity depends to a great extent on the selection and combination of HU and SUV thresholds[1]. Due to the lack of consensus on the most appropriate HU and SUV thresholds to quantify BAT volume and activity in humans, studies have shown different levels of BAT volume and activity in cohorts with similar characteristics [10,11]. Consequently, since optimal thresholds are not known, current available data on human BAT volume and activity are speculative at best. Moreover, the use of different HU and SUV thresholds hampers comparability across studies.

Recently, an expert panel launched a set of recommendations for conducting 18F-FDG-PET/CT analysis of human BAT (Brown Adipose Reporting Criteria in Imaging Studies, BARCIST 1.0) [1]. BARCIST 1.0 recommends using a HU range between -190 and -10, and a SUV threshold of [1.2/(lean body mass (LBM)/body mass (BM))]. The impact of using the BARCIST 1.0 HU and SUV thresholds compared to the most commonly used protocols to quantify BAT volume and activity is currently unknown since, to our knowledge, no studies using these specific thresholds have been reported yet. It is of interest to better understand the relative differences obtained with BARCIST 1.0 thresholds compared to the currently most commonly used thresholds in literature for populations with different age and BMI.

Therefore, in the present study we aimed to compare and quantify BAT volume and activity following BARCIST 1.0 recommendations against the most commonly used HU and SUV thresholds in three different cohorts of men including young lean adults, young overweight/obese adults, and middle-aged overweight/obese adults.

MATERIAL AND METHODS

Participants

A total of thirty men from three independent cohorts were included in this prospectively designed study. Participants' characteristics are shown in Table 1. The study cohorts were: (i) 10 young lean adults (21-29 years old; BMI 19-24 kg/m^2, white Caucasians) [12], (ii) 10 young overweight/obese adults (18-25 years old; BMI 25-35 kg/m^2) [13], and (iii) 10 middle-aged overweight/obese adults (35-53 years old; BMI 26-30 kg/m^2). The study conducted in young overweight/obese adults was approved by the Human Research Ethics Committee of both University of Granada (n°924) and Servicio Andaluz de Salud (Centro de Granada, CEI-Granada). The Medical Ethical Committee of the Leiden University Medical Center approved the other study cohorts (2473 and NCT02294084). All volunteers provided written informed consent before participation. All studies were performed in accordance with the Declaration of Helsinki.

Systematic review on most commonly used thresholds

To identify the most commonly used HU and SUV thresholds for 18F-FDG-PET/CT scans in

BAT research, we conducted a systematic literature search on MEDLINE (from January 1st 2007 to March 10th 2017) for studies reporting human BAT volume and activity. We used the Medical Subject Heading (MeSH) terms "Adipose Tissue, Brown" in combination with type of population (men, women, and adults) and instrument used ("PET/CT", "PET-CT", and "18-FDG"). We excluded human studies that did not use 18F-FDG-PET/CT scans to assess BAT volume and activity, studies conducted in animal models or not written in English, and reviews. In addition, we searched the reference lists of all identified relevant publications. No restrictions were considered regarding study design (cross sectional, case-control, cohort study) or data collection (prospective or retrospective). To avoid duplicate data, we identified articles that included the same group of participants by reviewing inter-study similarities in any of the following characteristics: country in which the study was conducted, investigators who performed the study, source of patients, recruitment period, and inclusion criteria. When the same investigators reported results obtained on the same group of patients in several publications, only the first published study was included.

Cooling protocol

To activate BAT, we applied personalized cooling protocols prior to 18F-FDG-PET/CT scans. Slightly different water-cooling methods were used between the different cohorts due to differences in local equipment and protocols. In the young lean and middle-aged overweight/obese cohorts two water-perfused temperature-controlled mattresses (Blanketrol III, Cincinnati Sub-Zero Products, Cincinnati, OH, USA) were used, as previously described [12]. In short, participants were sandwiched between two water-perfused mattresses starting at a temperature of 32°C, which was subsequently gradually decreased. When shivering occurred (after 30-40 min), temperature was raised by 3-4°C and a stable cooling period of 2 h started. In the cohort of young overweight/obese adults a water perfused vest (Polar Products Inc., Ohio, USA) was used [14]. Shivering threshold was determined 48-72 h before the 18F-FDG-PET/CT scan. Immediately before the 18F-FDG-PET/CT scan, participants wore the water perfused vest for 2 h set at approx. 4°C above the temperature that caused the onset of shivering. In all three studies, after 1 h of stable cooling 18F-FDG was injected (Table 1) and after 2 h of cooling 18F-FDG-PET/CT imaging was performed.

Body composition

Fat mass and lean body mass were measured by Dual Energy X-ray Absorptiometry (DEXA) in the three cohorts (Table 1).

18F-FDG-PET/CT scan

Cold-induced BAT volume and activity were assessed by 18F-FDG-PET/CT scanning. Table 1 shows details of the PET/CT scanners as well as doses of 18F-FDG injected in each study cohort. In the three studies, for the CT acquisition a peak kilovoltage of 120 was applied, while for the PET acquisition a scan time of 6 min per bed position was set. In total, 6 bed positions were scanned for the young lean and middle-aged overweight/obese adults (from top of the head to pelvis) and 2 bed positions for the young overweight/obese adults (from atlas vertebra to mid-chest, which was a requirement set by the Human Research Ethics Committee to limit radiation burden).

Quantification of human BAT volume and activity

PET/CT images were analyzed using the Beth Israel plugin for FIJI [2] software by two trained researchers [15]. Disagreements between both researchers were arranged in a consensus meeting. The regions of interest (ROIs) were semi-automatically outlined from atlas vertebrae (Cervical 1) to thoracic vertebrae 4 (see Figure 1) using a 3D-Axial technique[11]. The ROIs were placed in this range because (i) this region predominantly shows BAT activity and (ii) it is the region that is consistently scanned in all three study

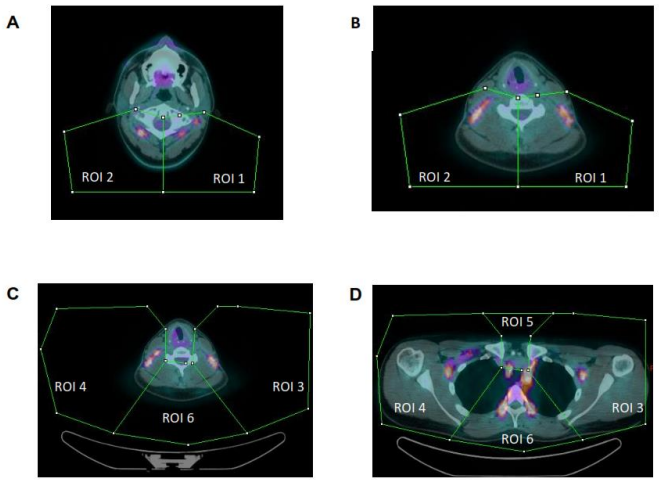

Figure 1. 3D-Axial technique: definition of the region of interest (ROI) drawn in the 18F-fluorodeoxyglucose-Positron Emission Tomography/Computed Tomography images of a representative individual. A. ROI 1 and 2 in atlas. B. ROIs 1 and 2 in the end cervical vertebrae 6. C. ROI 3, 4 and 6 in the beginning of cervical vertebrae 7. D. ROI 3, 4, 5 and 6 in thoracic vertebrae 4.

cohorts. Regions such as mouth, nose, or thyroid were not included to avoid potential false positives within the ROIs. We calculated the standardized uptake value (SUV) as [18F]-FDG uptake (kBq/mL)/(injected dose [kBq]/patient weight [g])]. We defined BAT volume, SUVmean, BAT metabolic activity, SUVmax and SUVpeak following BARCIST 1.0 criteria [1]. In short, BAT volume was calculated as the sum of the volumes identified as BAT in each ROI (1 to 6, see Figure 1). SUVmean was calculated by the weighted average of SUVmean derived from each ROI (1 to 6). SUVpeak was the highest average SUV in a 1 ml spherical volume. This sphere may, or may not, be centered on the highest SUVmax over all ROIs (1 to 6). We also drew a ROI on the descending aorta as reference tissue (Table 1) [1]. To quantify BAT, we applied the criteria of BARCIST 1.0, in addition to the three most used combinations of thresholds detected in the systematic review (see below).

Statistical analysis

Differences across HU and SUV thresholds were analyzed using analysis of variance (ANOVA) with Bonferroni adjustments for post-hoc comparisons. Separate analyses were conducted for each study cohort. The inter-observer reliability was assessed using Lin´s concordance coefficient (LCC) [16]. All analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM SPSS Statistics, IBM Corporation) and the level of significance was set to ≤0.05. Data are presented as mean ± standard deviation unless otherwise stated.

RESULTS

Characteristics of participants

Table 1 summarizes the characteristics of the participants and the study conditions in the three cohorts.

Table 1. Characteristics of participants and study conditions of the three cohorts

	Young lean adults (N=10)	Young overweight/obese adults (N=10)	Middle-aged overweight/obese adults (N=10)
Age (years)	25 ± 3	22 ± 2	42 ± 6
Height (m)	1.85 ± 0.05	1.78 ± 0.09	1.80 ± 0.05
Weight (kg)	76.0 ± 7.3	92.9 ± 16.1	89.9 ± 7.6
BMI (kg/m^2)	22.2 ± 1.6	29.0 ± 3.4	27.8 ± 1.3
Waist-to-hip ratio	0.87 ± 0.04	0.90 ± 0.08	0.96 ± 0.04
Fat mass (kg)	13.4 ± 4.2	31.7 ± 9.7	24.7 ± 6.5
Lean body mass (kg)	59.6 ± 5.9	56.2 ± 7.3	61.9 ± 5.1
Fasting glucose (mmol/l)	4.2 ± 0.3	5.0 ± 0.4	4.7 ± 0.4
Study period	March 2013 to June 2013	October 2015 to November 2016	March 2015 to June 2016
Country	The Netherlands	Spain	The Netherlands
Cooling methodology	2 water-perfused blankets (Blanketrol III, Cincinnati USA)	1 water-cooling vest (Polar Products Inc., Ohio, USA) with light cool air conditioned room.	2 water-perfused blankets (Blanketrol III, Cincinnati USA)
Time of cold exposure prior PET/CT scan	Shivering test + 2 h to individualized mild cold	Shivering test (48-72 h before) + 2 h to individualized mild cold	Shivering test + 2 h to individualized mild cold
PET/CT scanner	Gemini TF64 PET/CT, Philips, Netherlands	Siemens Biograph 16 PET/CT, Siemens, Germany	Gemini TF64 PET/CT, Philips, Netherlands
DEXA scan	Lunar iDXA, GE Healthcare, UK	QDR 4500W, HOLOGIC, USA	Lunar iDXA, GE Healthcare, UK
18F-FDG (MBq)	152 ± 15	188 ± 11	107 ± 4
Ratio 18F-FDG (MBq) to BMI (kg/m2)	6.8 ± 0.3	6.5 ± 0.8	3.8 ± 0.3
SUV lean body mass 1 threshold (g/ml)	1.54 ± 0.09	1.98 ± 0.17	1.75 ± 0.15
SUVmean (g/ml) of descending aorta (reference tissue)	1.18 ± 0.17	1.62 ± 0.23	1.66 ± 0.29

Data are means and standard deviation. BMI: Body mass index; DEXA: Dual Energy X-ray Absorptiometry; 18F-FDG: 18F-fluorodeoxyglucose; MBq: Megabecquerel; PET/CT: positron emission tomography/computed tomography; SUV: standardized uptake value.

Systematic review: Selection of HU and SUV thresholds

After having read titles and abstracts, 344 studies were excluded from a total of 471; 131 were checked for relevance (full text); and 123 met the inclusion criteria. After excluding redundant studies, i.e. studies that used the same participants and images, a total of 116 studies were finally included (Table 5). Table 2 shows a summary of the HU and SUV thresholds used in the quantification of human BAT by 18F-FDG-PET/CT. We found nine different combinations of HU and SUV thresholds published twice or more, and 26 different combinations of HU and SUV thresholds published just once. HU and/or SUV thresholds were not mentioned in as many as 32 studies. We selected the three most frequently used combinations of HU and SUV thresholds (Table 2): HU: -250,-50; SUV: 2.0, HU: N.A.; SUV: 2.0 and HU: -180,-10; SUV: 1.5 for comparison with the BARCIST 1.0 criteria.

High inter-observer reliability was found regardless of thresholds applied

Lin's concordance coefficient (LCC) of BAT volume and in the three cohorts was above 0.950, except in one case (0.906; SUVpeak in overweight/obese adults; HU: NA, SUV: 2.0;

Table 2. Summary of Hounsfield units (HU) and standardized uptake value (SUV) thresholds used for quantification of human brown adipose tissue by 18F-FDG-PET/CT scans from January 1, 2007 to March 10, 2017.

Hounsfield Units	SUV ≥	No. of studies: 116
-250;-50	2.0	20
Not used/Not reported	2.0	10
-180;-10	1.5	7
-300;-10	2.0	4
-100;-10	1.0	3
-250;-10	2.0	3
-150;-30	1.5	2
-250;-50	SUV different at 2.0	7
-180;-10	SUV different at 1.5	2
Other combinations		26
Not reported	Not reported	32

Table 3. Lin´s concordance coefficient for the inter-observer reliability of BAT volume and activity by study cohort and by threshold of HU and SUV for quantification of BAT.

	BARCIST 1.0		HU: -180, -10; SUV: 1.5		HU: -250, -50; SUV: 2.0		HU: NA; SUV: 2.0	
Young normal-weight adults								
Volume	0.962	(0.858 - 0.990)	0.953	(0.833 - 0.987)	0.976	(0.908 - 0.994)	0.951	(0.827 - 0.987)
SUV_{mean}	0.980	(0.930 - 0.934)	0.964	(0.877 - 0.990)	0.982	(0.933 - 0.996)	0.968	(0.908 - 0.989)
SUV_{peak}	1.000		1.000		1.000		1.000	
Young overweight-obese adults								
Volume	0.990	(0.961 - 0.997)	0.993	(0.973 - 0.998)	0.993	(0.974 - 0.998)	0.986	(0.954 - 0.996)
SUV_{mean}	0.997	(0.989 - 0.999)	0.996	(0.985 - 0.999)	0.992	(0.975 - 0.998)	0.989	(0.961 - 0.997)
SUV_{peak}	0.996	(0.984 - 0.999)	0.996	(0.984 - 0.999)	0.996	(0.986 - 0.999)	0.996	(0.984 - 0.999)
Middle-aged overweight-obese adults								
Volume	0.996	(0.983 - 0.999)	0.992	(0.972 - 0.998)	0.999	(0.994 - 1.000)	0.983	(0.941 - 0.995)
SUV_{mean}	0.999	(0.998 - 1.000)	0.999	(0.998 - 1.000)	1.000	(0.999 - 1.000)	0.961	(0.951 - 0.997)
SUV_{peak}	0.983	(0.935 - 0.995)	0.982	(0.935 - 0.995)	0.993	(0.987 - 0.996)	0.906	(0.695 - 0.973)

Data are means and 95% confidence intervals.
Strength of the agreement: from 1.000 to 0.999 (almost perfect), from <0.999 to 0.95 (substantial), from <0.95 to 0.90 (moderate), <0.90 (poor).
BARCIST 1.0: HU:-190,-10; SUV: Individualized [1.2/(lean body mass/body mass)]; BAT: Brown adipose tissue; BMI: Body mass index; HU: Hounsfield units; SUV: Standardized uptake value.

Table 3). When BARCIST 1.0 criteria were applied, the LCC were 0.962-0.996, 0.980-0.996 and 0.983-1.000, for BAT volume, SUVmean, and SUVpeak, respectively (Table 3).

The combination of HU and SUV thresholds markedly affects estimation of BAT volume and activity across different cohorts

Representative images of BAT volume and activity of the three study cohorts resulting from different HU and SUV threshold combinations are shown in Figure 2. Firstly,

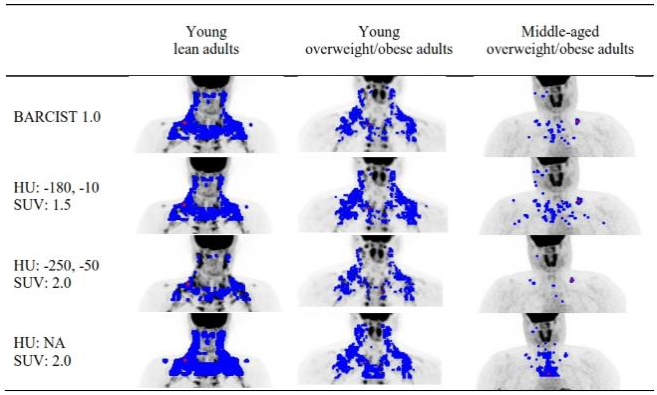

Figure 2. Representative images of brown adipose tissue volume and activity of the three study cohorts by threshold of Hounsfield units (HU) and standardized uptake value (SUV). Blue dots indicate BAT volume and red dots indicate maximal BAT activity (SUV max). BMI: Body mass index; HU: Hounsfield units; NA: Not applied; SUV: Standardized uptake value.

we determined the quantitative effect of the four threshold combinations on BAT volume in young lean adults (Fig. 3A), young overweight/obese adults (Fig. 3B), and middle-aged overweight/obese adults (Fig. 3C). Compared to the BARCIST 1.0 criteria, higher BAT volumes were consistently observed using the HU: NA; SUV: 2.0 criteria in young lean adults [+155%; 249 (186, 312) ml; mean (95% CI)], young overweight/obese adults [+207%; 244 (114, 374) ml], and middle-aged overweight/obese adults [+124%; 106 (42, 170) ml]. In contrast, BAT volumes estimated with the HU: -250,-50; SUV: 2.0 criteria compared to the BARCIST 1.0 criteria, yielded significantly smaller values in young lean adults [-57%; 92 (79, 106) ml], young overweight/obese adults [-42%; 49 (29, 70) ml], and middle-aged overweight/obese adults [-54%; 46 (28, 64) ml] (see Table 4). No significant differences were observed between BAT volumes estimated by HU: -180,-10; SUV: 1.5 criteria compared to BARCIST 1.0 criteria in young lean adults [0%; 0 (-5, 5) ml], however, we found higher BAT volumes in young overweight/obese adults [+40%; 47 (24, 69) ml] and middle-aged overweight/obese adults [+45%; 38 (18, 58)].

HU and SUV thresholds moderately affect estimation of BAT activity (SUVmean) across different cohorts

We next determined the effect of the four threshold combinations on SUVmean in young lean adults (Fig. 3D), young overweight/obese adults (Fig. 3E), and middle-aged overweight/obese adults (Fig. 3F). Compared to the BARCIST 1.0 criteria, higher SUVmean were observed using the HU: -250,-50; SUV: 2.0 criteria in young lean adults [+18%; 0.8 (0.9-0.6) g/ml] and in middle-aged overweight/obese adults [+13%; 0.3 (0.2-0.5) g/ml]. In young lean adults, a lower SUVmean was estimated using the HU: NA; SUV: 2.0 criteria compared to BARCIST 1.0 criteria [-9%; 0.4 (0.2, 0.6) g/ml]. Lower BAT activity was detected using HU: -180,-10; SUV: 1.5 criteria against BARCIST 1.0 criteria in young overweight/obese adults [-14%; 0.5 (0.4, 0.7) g/ml] and middle-aged overweight/obese adults [-11%; 0.3 (0.2-0.4) g/ml] (Table 4).

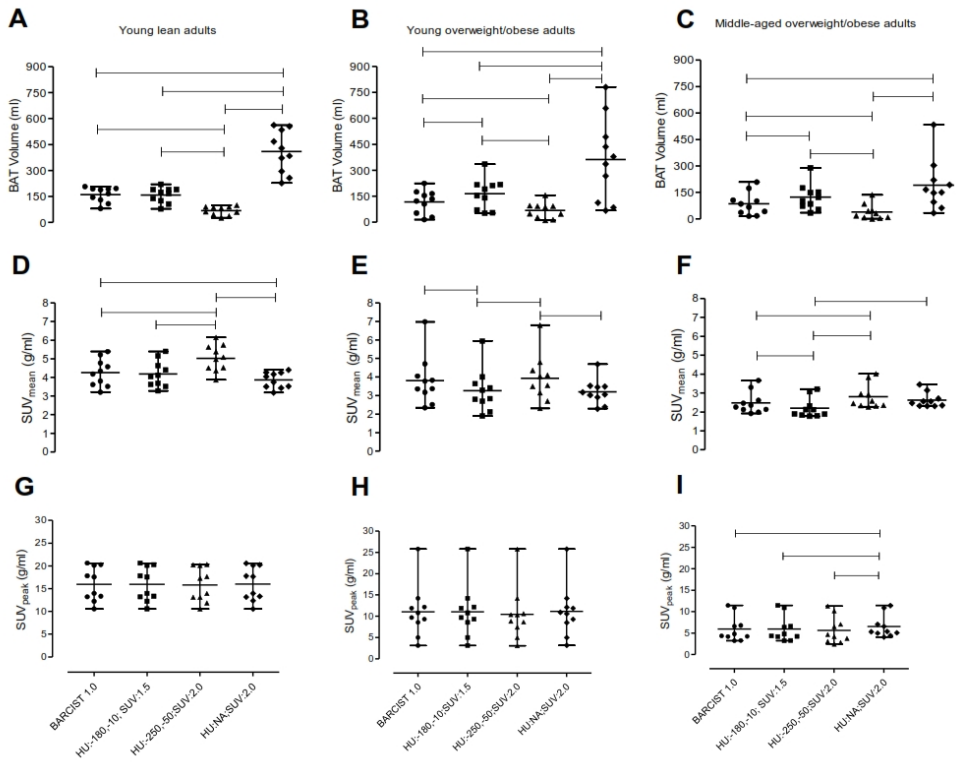

Figure 3. Brown adipose tissue (BAT) volume and activity determined by various thresholds of Hounsfield unit (HU) and Standardized uptake value (SUV) for three study cohorts. BAT volume (A-C), SUVmean (D-F), and SUVpeak (G-I) were determined in young lean adults (A, D, G), young overweight/obese adults (B, E, H), and middle-aged overweight/obese adults (C, F, I). Data are means and standard deviation (n=10 per cohort). Significant differences between thresholds are indicated by parallel horizontal bars (all P≤0.05). BARCIST 1.0: HU:-190,-10; SUV: Individualized [1.2/(lean body mass/body mass)]; BMI: Body mass index; NA: Not applied.

HU and SUV thresholds slightly affect estimation of maximal BAT activity (SUVpeak) values across different cohorts

In addition, we determined the effect of the four threshold combinations on SUVpeak in young lean adults (Fig. 3G), young overweight/obese adults (Fig. 3H), and middle-aged overweight/obese adults (Fig. 3I). We found similar results in the SUVpeak values (all P>0.05), however, there were significant differences in the middle-aged overweight/obese adults, where SUVpeak values calculated with HU: NA; SUV: 2.0 criteria were higher than calculated with BARCIST 1.0 criteria [+10%; 0.6 (0.2, 0.9) g/ml]. Figure 3 shows means and 95% CI of BAT volume and activity determined by various thresholds of HU and SUV for the three study cohorts.

DISCUSSION

To our knowledge, the present study is the first empirical investigation to compare and quantify BAT volume and activity following BARCIST 1.0 criteria against the most used HU and SUV thresholds in three independent cohorts of men of different age and BMI. We observed that BAT volume calculated using the HU: NA; SUV: 2.0 criteria may differ up to 2.6-fold, 3.1-fold, and 2.2-fold from the BAT volume calculated based on BARCIST 1.0 criteria in young normal-weight, young overweight/obese, and middle-aged

overweight/obese adults, respectively. Similarly, BAT activity (expressed as SUVmean), based on BARCIST 1.0 criteria differed up to 18% and 13% when compared with BAT activity based on the HU: -250,-50; SUV: 2.0 criteria, for young lean adults and middle-aged overweight/obese adults, respectively. On the other hand, differences were not significant when maximal BAT activity was expressed as SUVpeak for young cohorts, while we found that SUVpeak based on the HU: NA; SUV: 2.0 was 10% higher than calculated with BARCIST 1.0 criteria in middle-aged overweight/obese adults. The findings of this study show that human BAT volume and to a lesser extent BAT activity (expressed as SUVmean) depend largely on the selected HU and SUV thresholds.

Moreover, we observed that the various threshold combinations similarly influenced BAT volume and BAT activity (SUVmean) in the three cohorts independently of age, BMI or different methodologies applied to activate and measure BAT [17].

HU and SUV thresholds markedly affect BAT volume across different cohorts

The scientific community has been speculating about the volume of BAT that is present in adult men, and showed mean values of approx. 70 ml [3], 100 ml [2,18], 300 ml [19], and 450 ml. This highlights the knowledge gap regarding the amount of BAT present in adults and that maximizing its volume and metabolic activity could impact human physiology. We found that the highest BAT volumes were consistently observed with those thresholds that did not apply a criterion for HU (i.e. HU: NA, SUV: 2.0). Because the gold standard to quantify BAT is the 18F-FDG-PET in combination with CT, it is evident that a threshold of HU should be applied, although the most appropriate threshold of HU remains to be elucidated. We observed lower BAT volumes with the most frequently used threshold combination in literature (HU: -250,-50, SUV: 2.0) compared to BARCIST 1.0. These differences could be based on the fact that BARCIST 1.0 uses an HU range starting at -10 while the most commonly protocol used an HU range starting at -50, albeit it is known that the range of adipose tissue in the CT images starts at an HU value of -10 [11,20]. Moreover, it was recently shown [21] that the density of the BAT might change after a cold exposure, especially in the range from -50 to -10. Actually, it is not feasible to distinguish BAT from WAT or other tissues using exclusively CT criteria. Thus, more studies are needed to increase our understanding of BAT density measured by a CT scan [22].

The main advantage of the BARCIST 1.0 recommendations is the inclusion of an individualized SUV threshold adapted to the individual's lean body mass. Estimated BAT volumes did not differ between BARCIST 1.0 and HU: -180,-10; SUV: 1.5 criteria in the young lean adults, probably because the SUV criteria were virtually identical using both thresholds (1.54±0.09 vs. 1.5 g/ml, respectively). Nevertheless, we found higher estimated BAT volume with HU: -180,-10; SUV: 1.5 compared to BARCIST 1.0, because the SUV threshold applied in BARCIST was higher for young overweight/obese adults and middle-aged overweight/obese adults (1.98±0.17 g/ml and 1.75±0.15 g/ml, Table 1). Therefore, the relative changes in BAT volume are largely influenced by the use of a SUV threshold corrected by lean body mass [1,11].

HU and SUV thresholds moderately affect BAT activity (SUVmean) across different cohorts

SUVmean is the average SUV within a volume, therefore, if BAT volume differs due to HU or SUV thresholds in the three cohorts, SUVmean is also expected to differ by the thresholds selection. Indeed, SUVmean is not consistent across studies as it is influenced by many methodological factors as described previously[17] such as cooling protocols or instruments, as well as study populations[8,9]. We showed that when a SUV individualized threshold was used and the SUV values are alike (such as HU: -180,-10;

Table 4. Absolute and relative (%) differences between thresholds in brown adipose tissue volume and activity by study cohort.

Young lean adults		Mean	95%CI	Mean	95%CI	Mean	95%CI	
BARCIST 1.0 vs. HU: NA; SUV: 2.0	Absolute	249***	186 312	0.4**	0.2 0.6	0	0	0.1
	%	+155		-9		-0.2		
BARCIST 1.0 vs. HU: -250, -50; SUV: 2.0	Absolute	92**	79 106	0.8***	0.6 0.9	0.1	0	0.2
	%	-57		+18		-0.8		
BARCIST 1.0 vs. HU: -180, -10; SUV: 1.5	Absolute	0	-5 5	0	0 0.1	0	0	0
	%	0		0		0		
HU: -250, -50; SUV: 2.0 vs. HU: NA; SUV: 2.0	Absolute	341***	267 415	1.2***	0.9 1.4	0.2	0	0.3
	%	+500		-23		+1		
HU: -250, -50; SUV: 2.0 vs. HU: -180, -10; SUV: 1.5	Absolute	92***	77 108	0.8***	0.6 1.0	0.1	0	0.2
	%	+135		-16		+1		
HU: -180, -10; SUV: 1.5vs. HU: NA; SUV: 2.0	Absolute	249***	186 311	0.4	0.1 0.6	0	0	0.1
	%	+155		-9		0		
Young overweight/obese adults								
BARCIST 1.0 vs. HU: NA; SUV: 2.0	Absolute	244**	114 374	0.6	0.1 1.1	0.1	0.3	0.1
	%	+207		-16		+1		
BARCIST 1.0 vs. HU: -250, -50; SUV: 2.0	Absolute	49**	29 70	0.1	0 0.3	0.6	0.2	1.1
	%	-42		+3		-6		
BARCIST 1.0 vs. HU: -180, -10; SUV: 1.5	Absolute	47**	24 69	0.5*	0.4 0.7	0	0	0
	%	+40		-14		0		
HU: -250, -50; SUV: 2.0 vs. HU: NA; SUV: 2.0	Absolute	294**	146 442	0.7*	0.3 1.2	0.7	0.2	1.2
	%	+430		-19		+7		
HU: -250, -50; SUV: 2.0 vs. HU: -180, -10; SUV: 1.5	Absolute	96***	64 128	0.7*	0.6 0.8	0.6	0.2	1.1
	%	+141		-17		+6		
HU: -180, -10; SUV: 1.5vs. HU: NA; SUV: 2.0	Absolute	198**	73 322	0.1	-0.3 0.4	0.1	0.1	0.3
	%	+120		-2		+1		
Middle-aged overweight/obese adults								
BARCIST 1.0 vs. HU: NA; SUV: 2.0	Absolute	106**	42 170	0.1	0.1 0.3	0.6*	0.2	0.9
	%	+124		+6		+10		
BARCIST 1.0 vs. HU: -250, -50; SUV: 2.0	Absolute	46**	28 64	0.3*	0.2 0.5	0.3	0.1	0.8
	%	-54		+13		-6		
BARCIST 1.0 vs. HU: -180, -10; SUV: 1.5	Absolute	38**	18 58	0.3*	0.2 0.4	0	0	0.1
	%	+45		-11		-0.7		
HU: -250, -50; SUV: 2.0 vs. HU: NA; SUV: 2.0	Absolute	152**	78 226	0.2	0 0.4	0.9*	0.3	1.5
	%	+384		-6		+16		
HU: -250, -50; SUV: 2.0 vs. HU: -180, -10; SUV: 1.5	Absolute	84***	60 108	0.6*	0.5 0.7	0.3	0.2	0.8
	%	+213		-21		+5		
HU: -180, -10; SUV: 1.5vs. HU: NA; SUV: 2.0	Absolute	68	13 123	0.4*	0.3 0.5	0.6*	0.3	0.9
	%	+55		+19		+10		

Data are means and 95% confidence intervals.
BMI: Body mass index; HU: Hounsfield units; BARCIST 1.0: HU:-190,-10; SUV: Individualized [1.2/(lean body mass/body mass)]; NA: Not applied; SUV: Standardized uptake value. * P≤0.05, ** P≤0.01, *** P≤0.001. See Figure 1 for graphical representation.

SUV: 1.5 criteria in young lean adults), then the SUVmean values are similar. However, we observed that an application of a higher SUV threshold results in higher SUVmean values, regardless of the HU thresholds applied or the cohort studied because the SUV threshold of 2.0 includes higher 18F-FDG uptake of BAT deposits. On the other hand, when an HU threshold was omitted (i.e. HU: NA; SUV: 2.0 criteria), the SUVmean values

were lower regardless of the cohort studied. In this case, all 18F-FDG present in the selected ROIs of the PET images is used for quantification of BAT activity even though part of 18F-FDG would represent uptake by other tissues that were not excluded for the analysis due to lack of HU criteria.

HU and SUV thresholds slightly affect maximal BAT activity (SUVpeak) values across different cohorts

BARCIST 1.0 recommends to report SUVpeak instead of SUVmax to avoid overestimation in the quantification of BAT activity [1], because SUVmax is the single highest uptake pixel in the ROI and could easily be an outlier whereas SUVpeak is the highest average SUV in a 1 cc spherical volume, thereby reducing a potential effect of outliers. This sphere may, or may not, be centered on the highest SUVmax. Letiner et al. [11] found that PET image resolution substantially influences observed BAT SUVmax but whether this resolution also affects SUVpeak in currently unknown. Compared to the BARCIST 1.0 criteria, we found similar SUVpeak values across thresholds in young lean men and overweight/obese men. However, differences were observed between the thresholds that did not use HU vs. all the other thresholds in middle-aged overweight-obese adults. This could be based on the lower amount of 18F-FDG injected with respect to the size/body weight of the participant [ratio between amount of 18F-FDG to BMI (6.8±0.3, 6.5±0.8, and 3.8±0.3 MBq/(kg/m2) in young lean adults, young overweight/obese adults, and middle-aged overweight/obese adults, respectively]. Therefore, the distribution of the tracer among the various tissues may partially explain this finding. In fact, lower doses of 18F-FDG, as used in the cohort of middle-aged overweight/obese adults, may increase noise in the image and, therefore, raise SUVpeak levels [1,8,9]. We found that SUVmax was located in BAT regions irrespective of the threshold used in young lean and young overweight/obese men.

However, in middle-aged overweight/obese men, SUVmax was found in an unexpected region when no threshold for HU was used. Therefore, omission of HU threshold may have resulted in an artificial SUVmax and consequently SUVpeak, especially in the middle-aged overweight-obese men who received a low dose of 18F-FDG. Similar differences between thresholds and cohort studies were found with SUVmax (data not shown). Therefore, in light of these findings, we support that SUVpeak is the most consistent BAT-related outcome between criteria in the three independently cohorts of adults.

Limitations

We quantified BAT in six different ROIs from cerebellum to thoracic vertebra 4 (Figure 1). Although most of the BAT detected in humans is localized in the areas covered by the selected ROIs [11], we may have missed BAT depots in axillary, paraspinal or abdominal adipose tissue located in anatomical areas beneath the thoracic vertebra 4 [11]. Our ROIs did not include mouth, nose, or thyroid to avoid false positive results, yet, results persisted when a single ROI from cerebellum to thoracic vertebrae 4 was drawn and when HU criteria were applied (data not shown). In addition, we do not know if these findings can be replicated when the SUV threshold of BARCIST criteria is used in combination with other ranges of HU.

Besides the selection of HU and SUV thresholds, quantification of human BAT volume and activity also depends on other methodological issues such as the cooling protocol, 18F-FDG-PET/CT methodology, segmentation software [17], tracer used (18F-FDG vs. 18F-FTHA [23]), intrinsic factors of the participants such as age, sex, or body composition, or extrinsic factors as outdoor temperature [24] or daily light [25] ,which limit comparisons across studies. To improve the understanding of human BAT measured by 18F-FDG-PET/CT, the reconstruction settings should be harmonized in a similar manner as proposed by the EANM guidelines for 18F-FDG tumor PET imaging[26]. In the present study, the PET/CT scans from young overweight/obese adults did not follow these guidelines, therefore we cannot guarantee that the recovery coefficients of the used

reconstructions are the same. Moreover, methodological differences between cohorts did not allow us to check whether differences between HU and SUV thresholds are of different magnitude. Also the use of different cooling techniques (cooling vests vs. mattresses) and protocols might have introduced some bias. This study included only healthy male adults. The results should be applicable to other populations, such as women and men with different fat distributions, although this should be verified by replication in other cohorts with larger sample size. Moreover, biopsies of BAT-classical depots would be necessary to identify the density window (in terms of HU) of this tissue in different populations.

CONCLUSIONS

BAT volume and activity as determined by 18F-FDG PET/CT highly depend on the quantification criteria used. Future human BAT studies should conduct sensitivity analysis with different thresholds in order to understand whether results are driven by the selected HU and SUV thresholds. According to our findings, when following an individualized cooling protocol, SUVpeak is the most consistent marker of maximal BAT activity across study cohorts independent of the HU and SUV threshold used, which may therefore facilitate comparisons across studies. The design of the present study precludes providing any conclusive threshold, but before more definitive thresholds for HU and SUV are available, we support the use of BARCIST 1.0 criteria to facilitate interpretation of BAT characteristics between research groups.

Table 5. Hounsfield Units and standardized uptake value thresholds and software used in BAT human studies from 1st of January 2007 to 10th of March 2017

Studies	Hounsfield units	Standardized uptake values	Software to quantify BAT
Hadi et al. 2007 [27]	NR	NR	NR
Kim et al. 2008 [28]	NR	NR	NR
Alkhawaldeh et al. 2008 [29]	NR	NR	NR
Basu et al. 2008 [30]	NR	NR	NR
Zukotynski et al. 2009 [31]	NR	NR	NR
Cypess et al. 2009 [2]	-250,-50	2.0	PET/CT viewer shareware
Van Marken et al. 2009 [3]	NR	NR	PMOD 2.85
Virtanen et al. 2009 [32]	NR	NR	NR
Saito et al. 2009 [5]	NR	NR	VOX-BASE
Au-Yong et al. 2009 [33]	Not used	Not used	Leonardo workstation
Paidisetty et al. 2009 [34]	NR	NR	NR
Lee et al. 2010 [35]	-250,-50	2.0	NR
Skarulis et al. 2010 [36]	NR	NR	MEDx image
Aukema et al. 2010[37]	NR	NR	Osirix DICOM viewer
Pfannenberg et al. 2010 [38]	-250,-50	2.0	NR
Park et al. 2010 [39]	NR	NR	NR
Zukotynski et al. 2010 [40]	NR	NR	NR
Garcia et al. 2010 [41]	NR	NR	NR
Rakheja et al. 2011[42]	NR	NR	NR
Ouellet et al. 2011 [43]	-100,-10	1.0	MIM software
Pace et al. 2011[44]	-250,-50	NR	Volumetrix
Orava et al. 2011 [45]	NR	NR	NR
Vijgen et al. 2011 [18]	NR	NR	NR
Lee et al. 2011 [46]	NR	2.0	NR
Lee et al. 2011 [47]	NR	2.0	NR
Jacene et al. 2011 [48]	NR	NR	NR
Huang et al. 2011 [49]	-250,-50	2.0	OsiriX 64-bit software
Yoneshiro et al. 2011 [50]	NR	NR	VOX-BASE workstation
Yilmaz et al. 2011 [51]	NR	NR	NR
Yoneshiro et al. 2012 [52]	NR	2.0	VOX-BASE workstation
Vrieze et al. 2012 [53]	-250,-50	2.0	Hybrid Viewer; HERMES
Muzik et al. 2012 [54]	-250,-50	2.0	NR
Vijgen et al. 2012 [55]	NR	NR	PMOD 2.85
Chalfant et al. 2012 [56]	NR	NR	SliceOmatic image software
Vosselman et al. 2012 [57]	-180,-10	1.5	PMOD 3.0
Cypess et al. 2012 [58]	-250,-10	2.0	PET/CT Viewer shareware
Ouellet et al. 2012 [59]	-100, -10	1.0	NR

Bredella et al. 2012 [60]	-250, -50	70%SUVmax	PET/CT Viewer shareware
Miao et al. 2012 [61]	-250, -50	NR	PET/CT Viewer shareware
Vogel et al. 2012 [62]	NR	NR	Osirix DICOM viewer
Schlögl et al. 2013 [63]	-250, -10	2.0	SPM8
Ahmadi et al. 2013 [64]	-87, -10	NR	NR
Banzo et al. 2013 [65]	NR	NR	NR
Carey et al. 2013 [66]	-180, -10	1.0	Extended BrillianceWorkstation Mathworks, Natick, MA
Ruth et al. 2013 [67]	-200,-10	2.0	IDL software
Lee et al. 2013 [68]	NR	2.0	Volumetrix
Pasanisi et al. 2013 [69]	-250, -50	NR	VOX-BASE workstation
Sugita et al. 2013 [70]	NR	2.0	PMOD
Van Rooijen et al. 2013 [71]	-150, -50	NR	VOX-BASE workstation
Yonsehiro et al. 2013 [72]	NR	2.0	VOX-BASE workstation
Yoneshiro et al. 2013 [73]	NR	2.0	VOX-BASE workstation
Yoneshiro et al. 2013 [74]	NR	2.0	NR
Orava et al. 2013 [75]	NR	NR	AMIDE software
Muzik et al. 2013 [76]	-250,-50	2.0	NR
Chen et al. 2013 [77]	NR	2.0	PMOD 3.0
Vosselman et al. 2013 [78]	-180, -10	1.5	Syngo MI workplace
Perkins et al. 2013 [79]	-250, -50	No limit	PET/CT Viewer shareware
Bredella et al. 2013 [80]	-250, -50	70%SUVmax	PMOD 3.0
van der Lans et al. 2013 [81]	-180, -10	1.5	PET/CT viewer software
Zhang et al. 2013 [82]	-250, -50	2.0	Hermes Hybrid Viewer
Admiraal et al. 2013 [83]	-250, -50	2.0	Hermes Hybrid Viewer
Admiraal et al. 2013 [84]	-250, -50	2.0	MIM software
Persichetti et al. 2013 [85]	-250, -50	2.0	PMOD
Vijgen et al. 2013 [86]	NR	NR	Hermes Hybrid Viewer
Boon et al. 2014 [87]	Not used	2.0	NR
Lee et al. 2014 [88]	-300,-10	2.0	Software built with IDL
Lee et al. 2014 [89]	-300, -10	2.0	syngo.via software
Jang et al. 2014 [90]	NR	1.5	NR
Chondronikola et al. 2014 [91]	-100, -10	1.0	Hybrid Viewer; HERMES
Schopman et al. 2014 [92]	-250,-50	2.0	NR
Blondin et al. 2014 [93]	-150,-30	1.5	Hermes Hybrid Viewer
Bakker et al. 2014 [12]	Not used	2.0	NR
Zhang et al. 2014 [94]	-250, -50	2.0	VOX-BASE workstation
Matsushita et al. 2014 [95]	Not used	2.0	NR
Vosselman et al. 2014 [96]	-180, -10	1.5	PET/CT Viewer shareware
Zhang et al. 2014 [97]	NR	NR	

Choi et al. 2014 [98]	-250,-50	2.0	Extended Brilliance Workspace
Bredella et al. 2014 [99]	-250, -50	70%SUVmax	PET/CT Viewer shareware
Orava et al. 2014 [100]	Not used	1.14	Vinci 2.54.0 software
Cao et al. 2014 [101]	NR	NR	NR
Hanssen et al. 2015 [102]	-180, -10	1.5	PMOD 3.0
Hanssen et al. 2015 [103]	-180, -10	1.5	PMOD 3.0
Hanssen et al. 2015 [104]	-180, -10	1.5	PMOD 3.0
Blondin et al. 2015 [105]	-150, -30	1.5	NR
Blondin et al. 2015 [23]	-150, -30	1.5	NR
Dinas et al. 2015 [106]	Not used	Not limit	NR
Vosselman et al. 2015 [107]	-180, -10	1.5	PMOD 3.0
Cypess et al. 2015 [108]	-250,-10	2.0	PET/CT Viewer shareware
Nirengi et al. 2015 [109]	-300, -10	2.0	VOX-BASE workstation
Carey et al. 2015 [110]	-180,-10	2.0	Extended BrillianceWorkstation
Wei et al. 2015 [111]	-150,-30	2.5	PET/CT viewer shareware
Butler et al. 2015 [112]	-250,-50	2.0	NR
Raiko et al. 2015 [113]	NR	NR	NR
Wang et al. 2015 [114]	-250,-50	2.0	Volume Viewer software
Puar et al. 2016 [115]	-180, -10	1.5	Inveon Research software
Hanssen et al. 2016 [116]	-180, -10	1.5	PMOD 3.0
Singhal et al. 2016 [117]	-250, -10	1.0 to 30	Fiji Software
Oxguven et al. 2016 [118]	-250,-50	2.0	Advantage Windows Workstation 4.5
Chondronikola et al. 2016 [119]	-190, -30	1.5	MIM software
Chondronikola et al. 2016 [120]	NR	NR	NR
Gifford et al. 2016 [121]	-200, -1	No limits	NR
Yoneshiro et al. 2016 [122]	Not used	2.0	NR
Ramage et al. 2016 [123]	-150, -30	2.0	PMOD 3.409
Bahler et al. 2016 [124]	NR	NR	NR
Bahler et al. 2016 [125]	-250,-50	2.0	Hermes Hybrid Viewer
Salem et al. 2016 [126]	-150, -5	2.0	NR
Bahler et al. 2016 [127]	-250,-50	2.0	Hermes Hybrid Viewer
Van der Lans et al. 2016 [128]	-180, -10	1.5	PMOD 3.0
Gatidis et al. 2016 [129]	NR	NR	NR
Nirengi et al. 2016 [130]	NR	NR	NR
Shao et al. 2016 [131]	-100, -10	1.0	TrueD system
Hibi et al. 2016 [132]	Not used	2.0	VOX-BASE workstation
Chen et al. 2016 [133]	-180, -10	1.5	NR
Lee et al. 2016 [134]	-300, -10	2.0	Software built with IDL
Becker et al. 2016 [135]	-250, -50	3.0	AW version 4.6, GE Healthcare

Takx et al. 2016 [136]	-250, -50	2.0	TrueD system
Shao et al. 2016 [137]	-250, -50	2.0	Syngo True D system
Muzik et al. 2016 [138]	-250, -50	2.0	AMIDE software
Blondin et al. 2016 [139]	-150, -30	1.5	NR
Blondin et al. 2017 [140]	NR	NR	NR
Gerngrob et al. 2017 [19]	-250, -50	2.0	"SYNGO" workstation (Siemens)
Hussein et al. 2017 [141]	-190,-30	2.0	NR
Yoneshiro et al. 2017 [142]	-300, -10	2.0	NR

NR: Not reported or cited in the study.

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Distribution of brown adipose tissue radiodensity in young healthy adults

CHAPTER 7

BACKGROUND

In 2009 a set of studies demonstrated that humans have metabolically active brown adipose tissue (BAT) upon cold exposure [1–3]. Because of its energy-burning capacity, BAT has been postulated as a possible tool to combat obesity and other comorbidities [4,5]. To date, the most used technique to quantify human BAT after acute cold exposure [6] is the injection of a single dose of 18F-fluorodeoxyglucose (18F-FDG) before performing a positron emission tomography (PET) combined with X-ray computed tomography (CT) scan [7].

18F-FDG-PET provides information about 18F-FDG uptake by metabolically active tissues and is expressed as standardized uptake value (SUV). After entering into the cells, 18F-FDG is phosphorylated by hexokinase. Because it cannot be further metabolized, it becomes entrapped and can be visualized using PET [7]. Human studies apply a minimum threshold of SUV to consider the metabolic activity itself as BAT, but only using 18F-FDG-PET scan is inaccurate because the technique does not allow to identify tissues. Therefore, 18F-FDG-PET is commonly combined with CT, which uses rotating X-rays and tomographic reconstructions to visualize internal body structures [7]. A CT scan is able to distinguish different tissues based on the radiodensity, which is expressed as Hounsfield Units (HU). The radiodensity of adipose tissue ranges from -10 to -300 HU. The radiodensity of BAT ranges from -10 to -67 HU and that of WAT from -96 to -300 HU [8–10]. U Din et al. [11] showed that human BAT radiodensity increased from thermoneutral to cold conditions and this increase was negatively associated to adiposity markers.

To date, human BAT studies have applied more than 32 combinations of SUV and HU thresholds [12] and these combinations influence BAT volume and activity as quantified by 18F-FDG-PET/CT scans. In 2016, a panel of experts published a set of recommendations to perform and analyse human BAT studies using this technique [6], and concluded that the SUV threshold should be individualized (SUV IND) to the participant's body composition [6]. Additionally, they suggested a HU threshold from -10 to -190 for BAT detection and recommended that all studies should apply these thresholds in order to facilitate future comparisons. Nevertheless, it remains unknown whether SUV or HU thresholds influence 18F-FDG uptake by BAT quantification the most. Furthermore, to date, the most used HU threshold applied in BAT quantification is from -50 to -250 HU [12], and several studies showed with biopsies that BAT density corresponds with the radiodensity measured with HU in the range from -10 to -50 [8–10]. It is unknown how much BAT is not taken into account using that smaller threshold. Lastly, BAT volume and activity differ between sexes, as women have higher 18F-FDG uptake by BAT compared to men [13], and it is affected by body composition. However, whether any differences exist in terms of BAT radiodensity in men and women and between individuals with different body composition is also largely unknown (13).

Thus, the aims of the present study are: (i) to examine the influence of SUV and HU thresholds on the quantification of BAT; (ii) to identify how much BAT measured by 18F-FDG-PET/CT scan is lost when limiting the range between -10 and -50 HU; and (iii) to describe the distribution of BAT radiodensity by weight status and sex in young healthy individuals.

MATERIAL AND METHODS

Participants

A total of 125 healthy adults (43 men and 82 women) aged 18-25 years, participated in the present study (Table 1) [15]. All participants were non-smokers, were not enrolled in a weight loss program, had a stable body weight (changes <3 kg over the last 3 months), were not physically active (<20 minutes on <3 days/week), did not take any medication, had no acute or chronic illness, and were not pregnant. The study was conducted between October and November in 2015 and 2016 in Granada (Southern Spain). The study protocol and informed consent were performed in accordance with the

Declaration of Helsinki (revision of 2013), and were approved by the Human Research Ethics Committee of both University of Granada (n° 924) and Servicio Andaluz de Salud (Centro de Granada, CEI-Granada). A written informed consent was obtained from all participants.

Personalized cooling protocol

The participants arrived at the lab in fasted condition (at least 6 h), after having slept as usual, having refrained from any moderate (for 24 h) or vigorous (for 48 h) physical activity, and having refrained from consumption of alcoholic or stimulating beverages (for 6 h) [13,16]. During the measurements, the participants wore standardized clothes (shorts, standard T-shirt, clo value=0.20) and were barefoot. A detailed description of the cooling protocol can be found elsewhere [13]. In brief, the participants rested 30 minutes in a warm room for acclimation (approx. 22.5°C). In order to determine their shivering threshold, the participants received a water-perfused cooling vest of which we reduced the temperature (Polar Products Inc., Ohio, USA) until shivering was self-reported and was visually observed by the researchers. 48-72 h later, participants underwent a personalized cold exposure (3.8°C above their personal shivering threshold) during 2 h using the cooling vest while sitting in a mild cold-air room (19.5-20°C). After the first hour of cold exposure, we administered 18F-FDG intravenously (185 MBq: ~2.78 MBq/kg), and we increased the water temperature by 1°C to avoid shivering. After the second hour of cold exposure, we performed the PET/CT scan (Siemens Biograph 16 PET/CT, Siemens Germany). Two bed positions were scanned from approximately atlas vertebrae to thoracic vertebrae 6.

PET/CT analysis

The PET/CT images were analyzed using the Beth Israel plugin for FIJI [1]. The regions of interest (ROIs) were semi-automatically outlined from atlas vertebrae (Cervical 1) to thoracic vertebrae 4 using a 3D-Axial technique [18]. We established the cervical region from atlas vertebrae to C7 (Figure 9A) and thoracic region from C7 to Th4 (Figure 9B). Regions such as mouth, nose, or thyroid were not included to avoid potential false positives within the ROIs [12]. We calculated the SUV as 18F-FDG uptake (kBq/mL)/(injected dose [kBq]/patient weight [g])]. We defined BAT volume, SUVmean, and SUVpeak following BARCIST 1.0 criteria [(SUV IND= (1.2/(lean body mass/body mass)), and we defined BAT metabolic activity as the product of BAT volume and SUVmean [6]. The Beth Israel plugin for FIJI software has the option to export a csv file with the single value of HU and SUV for every active pixel inside the ROIs. We quantified BAT volume and activity in every single range of HU (from 0 to -1, from -1 to -2, and so on until -300). Once we identified all the pixels located in the range of HU, we applied different SUV and HU thresholds. Firstly, we used different SUV thresholds (1.5, 2.0, 2.5, 3.0 and IND) with a fixed range of HU (-10, -300) for quantification of BAT volume and activity (SUVmean). Secondly, we used a fixed SUV threshold (SUV IND) but we modified the ranges of HU (-10, -190; -10, -180; -50,-190; and -50, -250) and we quantified again BAT volume and activity for every single combination. We established as reference BAT volume and activity as calculated with SUV IND as recommended by the BARCIST 1.0 criteria [6] and the range from adipose tissue (from -10 to -300 HU). We then averaged the number of pixels in every range of HU to obtain the average of HU. The PET/CT scans were visually and carefully examined to detect 18F-FDG uptake in BAT-specific depots. Participants were categorized as PET+ when BAT volume was ≥5 mL and 18F-FDG uptake was clearly apparent, and as PET- when BAT volume was <5 mL and there were no signs of 18F-FDG uptake by BAT [13,19].

Body composition

The body composition was measured by Dual Energy X-ray Absorptiometry (DEXA) scan (HOLOGIC, Discovery Wi). We measured the participants' weight and height without shoes and wearing the standard clothes using a SECA scale and stadiometer (model

799, Electronic Column Scale, Hamburg, Germany), and we calculated their BMI (kg/m^2). The participants were categorized as normal-weight (BMI ≥18.5 and <25 kg/m^2), overweight (BMI ≥25 and <30 kg/m^2) and obese (BMI ≥30 kg/m^2) [20]. Fat mass index (FMI) was calculated as kilograms (kg) of body fat divided by squared height in m^2, and lean mass index (LMI) was calculated as lean body mass in kg divided by squared height in m^2.

Statistical analysis

The descriptive characteristics of the study sample are presented as mean and standard deviation (SD) unless otherwise stated. To examine the influence of SUV and HU thresholds on the quantification of BAT, we conducted linear regression analysis with BAT volume and activity quantified by SUV IND; HU: -10, -300 as independent variable and BAT volume and activity quantified by different combination of HU and SUV thresholds as dependent variables. The analyses were conducted in separate regression models. To identify how much BAT measured by 18F-FDG-PET/CT scan is located in the range between -10 and -50 HU, we quantified the amount of BAT volume for every single range of HU. After that, we calculated the percentage of BAT volume and metabolic activity for every range of HU respect to the total amount. To study the interaction effect of the HU threshold with BMI or sex on the adipose tissue (BAT and WAT) volume, as well as on BAT volume and activity, we conducted a two-way analysis of variance (ANOVA) with repeated measurements. We introduced the amount of BAT calculated every 10 HU (for instance, from 0 to -10, from -11 to -20 HU and so on; 30 levels) as within-subject factor and weight status (underweight, normal weight, overweight, obese) or sex as between-subjects factors. Moreover, we calculated the mean value of HU with the different combination of threshold selected for the regression analyses. All analyses were performed using the IBM SPSS Statistics for Windows version 22.0 (Armonk, NY: IBM Corp), and the level of significance was set to P<0.05.

RESULTS

SUV rather than HU threshold influences BAT outcomes

First, we confirmed that 18F-FDG uptake by BAT significantly changed when both SUV and HU were modified in comparison to the reference criteria (SUV IND; HU:-10,-300) in the whole sample (Fig. 1) [12].

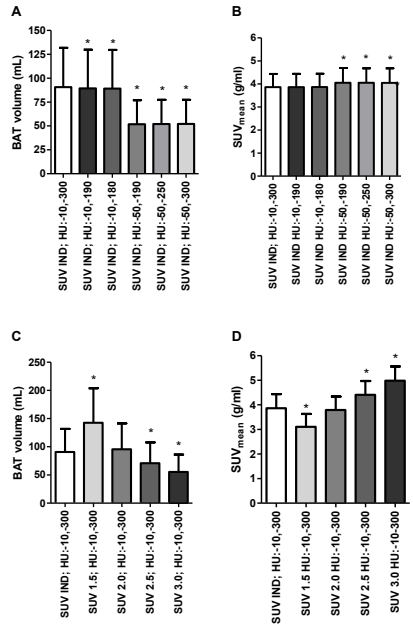

Figure 1. Differences between the most commonly published combinations of Hounsfield unit (HU) and standardized uptake value (SUV) criteria to quantify brown adipose tissue (BAT) volume (panel A and C) and activity (panel B and C) in 123 young healthy adults. White bars represent the individualized (IND) SUV threshold (i.e. considering lean body mass) with the wider range of HU as reference criterion. A, BAT volume quantified with the SUV IND threshold but modifying the range of HU. B, BAT activity, as SUV mean, quantified with the SUV IND threshold but modifying the range of HU. C, BAT volume quantified with the same HU range but modifying the SUV threshold. D, BAT activity, as SUV mean, quantified with the same HU range but modifying the SUV threshold. * P≤0.001 different from reference. Data are presented as mean and 95% confident interval.

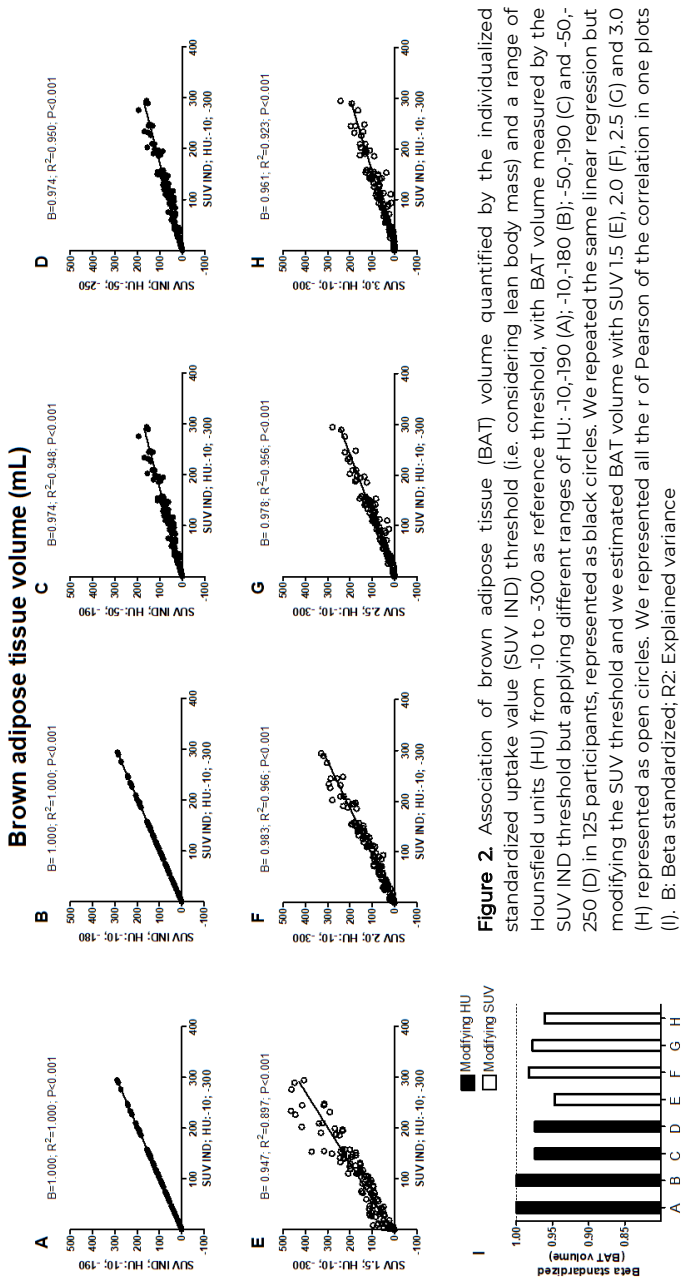

Figure 2. Association of brown adipose tissue (BAT) volume quantified by the individualized standardized uptake value (SUV IND) threshold (i.e. considering lean body mass) and a range of Hounsfield units (HU) from -10 to -300 as reference threshold, with BAT volume measured by the SUV IND threshold but applying different ranges of HU: -10,-190 (A); -10,-180 (B); -50,-190 (C) and -50,-250 (D) in 125 participants, represented as black circles. We repeated the same linear regression but modifying the SUV threshold and we estimated BAT volume with SUV 1.5 (E), 2.0 (F), 2.5 (G) and 3.0 (H) represented as open circles. We represented all the r of Pearson of the correlation in one plots (I). B: Beta standardized; R2: Explained variance

Next, we examined the association of BAT volume quantified by SUV IND; HU:-10,-300 with BAT volume using a fixed SUV IND threshold but modifying the HU thresholds [HU: -10,-190 (Fig. 2A), HU:-10,-180 (Fig. 2B), HU: -50, -190 (Fig. 2C) and HU: -50, -250 (Fig. 2D)]. We repeated the same analysis using a fixed HU range (-10, -300) but modifying the SUV threshold [SUV: 1.5 (Fig. 2E), 2.0 (Fig. 2F), 2.5 (Fig. 2G) and 3.0 (Fig. 2H)]. In all regressions, we found positive and significant associations (all P≤0.001). However, we found that by modifying the HU thresholds the explained variance (R2) varied from 0.948 to 1.000, whereas modifying the SUV resulted in a variation in R2 between 0.883 and 0.938 in comparison to BAT volume quantification by the reference method. Moreover, we found that beta standardized of these linear regressions were lower when we modified SUV thresholds in comparison to HU thresholds (Fig. 2I). Similar results were found

Figure 3. Association of brown adipose tissue (BAT) activity (SUVmean) quantified by the individualized standardized uptake value (SUV IND) threshold (i.e. considering lean body mass) and a range of Hounsfield units (HU) from -10 to -300 as reference threshold, with BAT activity (SUVmean) measured by the SUV IND threshold but applying different ranges of HU: -10,-190 (A); -10,-180 (B); -50,-190 (C) and -50,-250 (D) in 125 participants, represented as black circles. We repeated the same linear regression but modifying the SUV threshold and we estimated BAT activity (SUVmean) with SUV 1.5 (E), 2.0 (F), 2.5 (G) and 3.0 (H) represented as open circles. We represented all the r of Pearson of the correlation in one plots (I). B: Beta standardized; R2: Explained variance.

in terms of BAT activity (SUVmean) (Fig. 3). It should be noted that we only found a perfect correlation between BAT was quantified using the criteria of SUV IND; HU:-10,-300 with BAT as quantified by the BARCIST proposal (SUV IND; HU:-10,-190) and with SUV IND; HU:-10,-180), for both BAT volume and activity (B=1.000; R2=1.000; P≤0.001, Fig. 2A and B and Fig. 3A and B). Similarly, the mean value of HU differed when the SUV or the HU threshold changed (Fig. 4A and B). The differences were especially higher when the HU threshold excluded the range from -10 to -50 HU. Moreover, we showed that the association of the mean value of HU and BMI was affected by excluding the range from -10 to -50 HU (Fig. 4C and D).

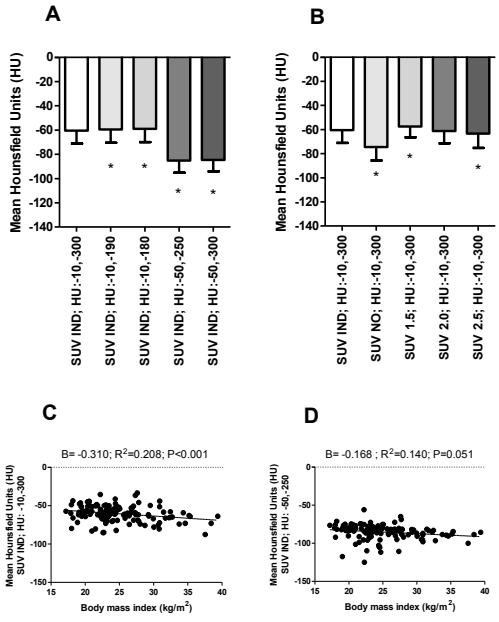

Figure 4. Differences between the most commonly published combinations of Hounsfield unit (HU) and standardized uptake value (SUV) criteria to quantify the average of brown adipose tissue (BAT) radio-density. Panel A, modifying the HU range, whereas panel B was modifying SUV thresholds. Panel C represents the association between body mass index with the average of BAT radio-density obtained by SUV: IND; HU: -10, -300, whereas panel D presents the association between body mass index with the average of BAT radio-density obtained by SUV: IND: HU: -50, -250. The linear regressions were adjusted by sex. B: Beta standardized; R2: Explained variance.

BAT is mainly present in the HU range between -10 and -100

We next studied in which HU range most BAT is present. To this end, we investigated the whole range of HU that is supposed to contain adipose tissue (both BAT and WAT; from -10 to -300 HU) and we applied the SUV IND threshold. We observed that BAT (as volume) was present mostly in the range from -10 to -150 HU (Fig. 5A and D), representing the 96.8% of the total BAT volume. We also calculated BAT activity (SUVmean) and BAT metabolic activity every 10 HU, finding similar results (Fig. 5B and C) in the range from -10 to -150 HU, which represent the 98.0% of the total metabolic activity of 18F-FDG uptake by BAT. Based on these data, the 43.2% of BAT volume was covered in the range -10 to -50 HU, and the 42.6% of BAT volume was covered in the range -51 to -100 HU (Fig. 5D). In addition, we calculated that the range -10 to -50 HU represented 41.4% of total metabolic activity of BAT and the range -50 to -100 HU represented 45.2% (Fig. 5E). At HU ≤ -150 only 5.2% of the total amount of BAT was present, with low 18F-FDG uptake by BAT (0.1% of total) (Fig. 5E).

BMI and sex affect the distribution of BAT radiodensity

We next calculated the percentage of the total amount of adipose tissue (without applying a SUV threshold, thus including both BAT and WAT), divided by different categories of BMI, in order to study the distribution of adipose tissue within the HU ranges. We observed that overweight and obese participants had a higher proportion of adipose tissue in the HU range from -75 to -300 HU, whereas underweight and normal weight participants had a higher proportion of adipose tissue with a higher density (Fig. 6A: P for HU*BMI ≤0.001). When we applied a SUV IND threshold for quantifying BAT, the differences in BAT volume radiodensity disappeared (Fig. 6B: P for HU*BMI =0.109). We also studied BAT activity (SUVmean), and we did not find differences between groups (Fig. 6C: P for HU*BMI=0.352). In our whole sample, we were unable to detect glucose uptake in BAT depots in 14 participants (PET-) (Table 1).

Table 1. Characteristics of participants (n=125).

Sex (women, n, %)	82, 65.6%
PET- (n, %)	14, 11.2%
Age (years)	21.9 ± 2.1
Lean mass index (kg/m2)	14.6 ± 2.5
Fat mass index (kg/m2)	35.8 ± 7.5
Body mass index (kg/m2)	24.9 ± 4.8
Underweight (n, %)	6, 4.8%
Normal weight (n, %)	71, 56.8%
Overweight (n, %)	28, 22.4%
Obese (n, %)	20, 16.0%

Data are presented as means and standard deviation.

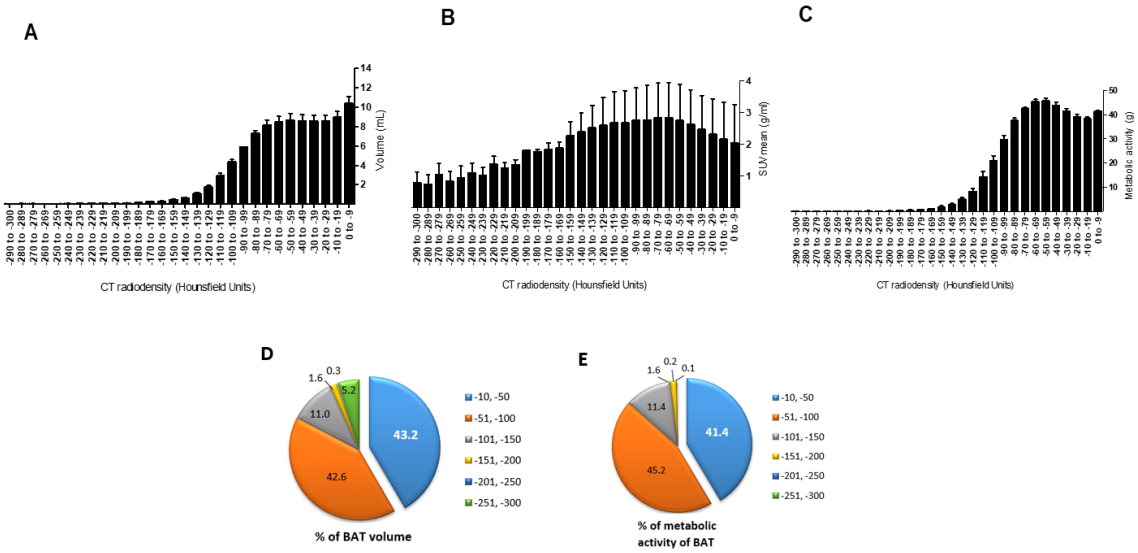

Figure 5. Amount of brown adipose tissue (BAT) by ranges of 10 Hounsfield units (HU) where adipose tissue is known to be present (from -10 to -300) in 125 participants. BAT was calculated using individualized standardized uptake value (SUV) threshold (i.e. considering lean body mass). A. BAT volume B. BAT activity (SUV mean). C. BAT metabolic activity. D. Percentage of BAT volume in different HU ranges respect to the total BAT volume. E. Percentage of BAT metabolic activity in different HU ranges respect to the total BAT metabolic activity. Data are represented as mean and standard deviation, otherwise indicated.

PET- participants had the same adipose tissue distribution in comparison to PET+ participants (Fig. 7A), but a different BAT radiodensity distribution (Fig. 7B: P for HU*PET<0.001). We therefore repeated the analyses excluding PET- scans and observed that the differences between adipose tissue radiodensity remained (Fig. 8A: P for HU*BMI ≤0.001). Moreover, after excluding PET-, there were differences in the distribution of BAT radiodensity between categories of BMI (Fig. 8B: P for HU*BMI= 0.004). Overweight and obese participants had a higher proportion of BAT radiodensity located in less denser ranges in comparison to normal weight and underweight participants, who had a higher proportion of BAT radiodensity in a denser range (from -10 to -50). There were no differences in terms of BAT activity (SUVmean) (Fig. 8C: P for HU*BMI= 0.126).

Next, we studied whether sex affects BAT radiodensity (Fig. 6D, E and F). Women had a higher percentage of adipose tissue in the value of radiodensity between -100 and -300 HU in comparison to men who had higher percentage of adipose tissue in a denser range (from -10 to -50 HU) (Fig. 6D: P for HU*sex≤0.001). In addition, when we applied the SUV IND threshold we observed that men had a higher proportion of BAT volume in comparison to women in denser ranges of HU (Fig. 6E: P for HU*sex=0.023). However, women showed higher proportion of BAT activity (SUVmean) in the whole range of HU than men (Fig. 6F: P for HU*sex≤0.001). All these results persisted when BMI was introduced as co-variable (data not shown).

Cervical BAT has higher radiodensity than thoracic BAT

We next analysed whether BAT radiodensity differed between the two most common human BAT depots, cervical vs. thoracic (supraclavicular, mediastinal and paravertebral) [21] (Fig. 9A-B) [18]. We observed that BAT volume of the cervical area was denser in comparison to thoracic BAT (Fig. 9C: P for HU*area≤0.001). These results persisted when we excluded PET- participants (Fig. 9D: P for HU*area ≤0.001) and when the analyses were adjusted by BMI or sex (data not shown).

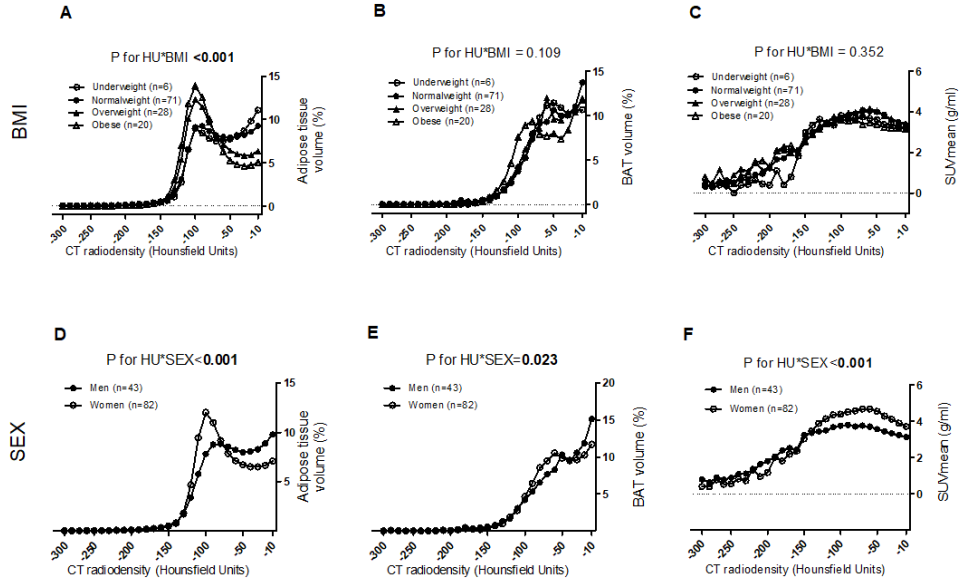

Figure 6. Patterns of adipose tissue (A and D), brown adipose tissue (BAT) volume (B and E) and BAT activity (C and F) by body mass index (BMI) and sex. A. Kinetics of fat volume without applying standardized uptake value (SUV) threshold by BMI categories D. Same as in A but by sex. B. Patterns of BAT volume after applying the individualized SUV threshold. E. Same as in D but by sex. C. Patterns of BAT activity after applying the individualized SUV threshold F. Same as in C but by sex. A., B., D., and E., are represented as percentage of the individual total volume. P for trend obtained from a bifactorial analysis of variance

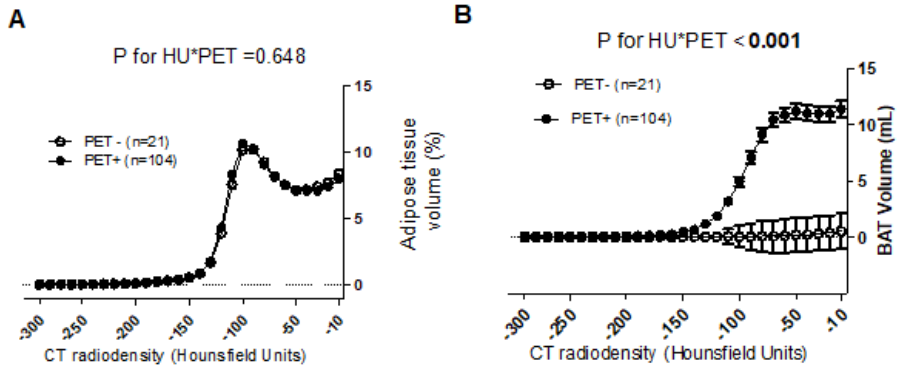

Figure 7. Kinetics of adipose tissue distribution in participants with positive and negative glucose uptake in brown adipose tissue (BAT) depots in the positron emission tomography images (PET+ and PET-, respectively). A., Percentage of adipose tissue distribution without applying any threshold of standardized uptake value (SUV). B., Percentage of BAT volume distribution quantified by SUV individualized to lean body mass in participants. P for two ways analysis of variance (ANOVA). Data are presented as mean and standard deviation.

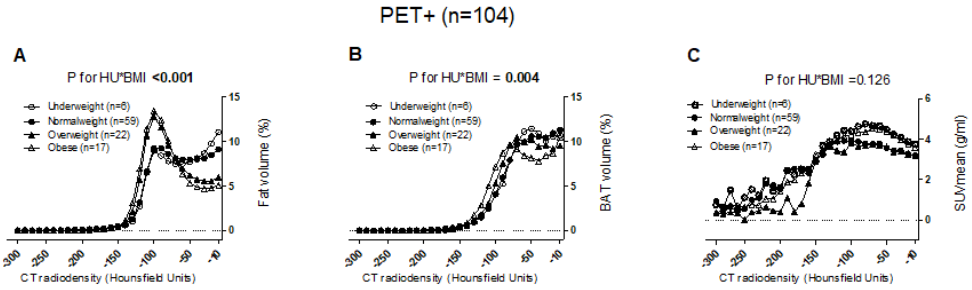

Figure 8. Patterns of adipose tissue (A), brown adipose tissue (BAT) volume (B) and activity (C) by BMI groups in PET+ participants. A., represent kinetics of volume of adipose tissue from the atlas vertebrae to thoracic vertebra 4 (Th4) without apply any standardized uptake value (SUV) in underweight, normal-weight, overweight and obese participants (n=125), B., represent kinetics of volume of BAT from the atlas vertebrae to Th4 after applied the SUV individualized threshold for the detection of BAT volume, C., represent kinetics of BAT activity as SUV mean. In all the graphs were included only participants who presented a positive visually glucose uptake (PET+; n=104). A., and B., are represented as percentage of total volume, whereas C is the SUVmean weighted in every 10 Hounsfields Units.

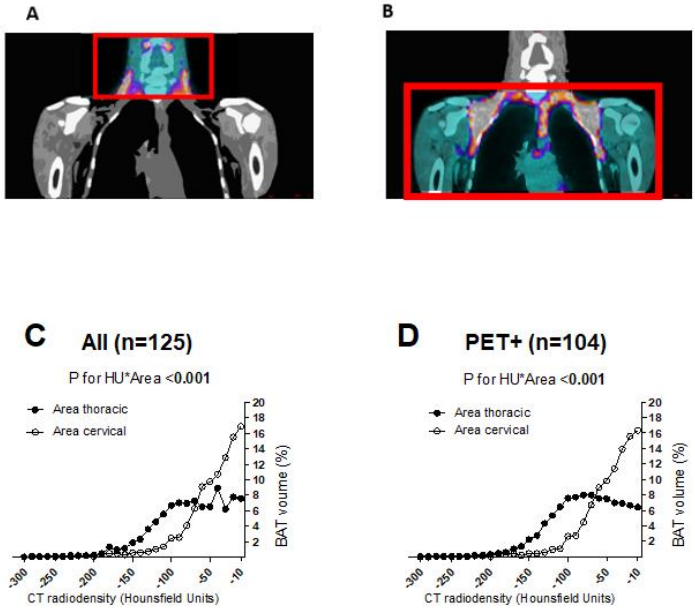

Figure 9. Distribution of brown adipose tissue (BAT) radio-density across ranges of 10 Hounsfield Unit (HU) by anatomic location (cervical vs. thoracic). A. Cervical region selected: from atlas cervical to cervical vertebrae 7) to do analysis of panel C. B. Thoracic region selected: from cervical vertebrae 7 to thoracic vertebrae 4 to do analysis of panel D. C. Percentage of BAT volume respect to the total amount of BAT volume in the cervical area, in every range of 10 HU in the whole sample. D. Percentage of BAT volume respect to the total amount of BAT volume in the thoracic area, in every range of 10 HU in PET+ participants. Data are represented as mean.

DISCUSSION

The present study shows that the SUV threshold influences BAT quantification measured by 18F-FDG-PET/CT scans more than the HU threshold. Furthermore, we observed that the range from -10 to -50 HU had the highest proportion of total human BAT volume (43.2%), which represents 41.4% of the total BAT metabolic activity in our cohort. Moreover, excluding the HU range between -10 and -50 affected also the mean value of HU. We furthermore showed that BAT volume radiodensity was not different between categories of BMI, as well as BAT activity (SUVmean). In addition, BAT radiodensity in women was less dense in comparison to men, although the BAT activity (SUVmean) was higher in all ranges of HU. Therefore, all future human studies using static 18F-FDG-PET/CT scans should include in their ranges of HU the range from -10 to -50 HU. This study also shows that the radiodensity of BAT located in the cervical area was mainly in the range from -10 to -50 HU.

Hounsfield Unit threshold

Recently, a consortium [6] suggested to use the SUV IND because 18F-FDG uptake is not equally distributed between tissues. Our results do support this idea, because modifying the SUV thresholds decreased the variance explained and the beta standardized of the linear regressions more in comparison to modifying the HU thresholds for BAT outcomes. In addition, BARCIST 1.0 proposed a range for the BAT radiodensity (from -10 to -190 HU) [6], which perfectly correlated with a criteria with bigger range of density (-10,-300 HU). Furthermore, we found that the 43.2% of the total amount of BAT is present in the range from -10 to -50 HU. Obviously, the most used HU threshold in literature (-50, -250 HU) is excluding this amount of BAT. Thus, we reason that these studies missed an important portion of the human BAT, probably cervical BAT.

On the other hand, U Din et al. [11] recently showed that the mean value of HU is inversely related to makers of adiposity (e.g. BMI and waist circumference). However, they quantified the mean value of HU with the most used threshold in literature (SUV: 2.0; HU: -50, -250). Here, we showed that the results of the associations between the mean value of HU with markers of adiposity are influenced by the fact of including the range from -10 to -50 HU, more than including the range from -190 to -250 HU or -190 to -300 HU. Therefore, future studies need to pay more caution to select the ranges of HU for BAT quantification, because it matters.

Differences in BAT radiodensity by BMI and sex

White adipose tissue (WAT) has the possibility to transdifferentiate in beige tissue by a process commonly named as browning [22]. During this process, the number of mitochondria, as well as the levels of uncoupling protein 1 (UCP1) increases in WAT [23]. Nothing is known about the radiodensity of beige tissue or whether radiodensity measured by CT scan is able to distinguish between brown, white and beige tissues. Based on mice studies [24], it can be postulated that radiodensity of beige tissues should be intermediate between BAT and WAT[25] “neither BAT, nor WAT is something in between”; however, further studies are needed to verify this in humans.

In 2012, Cypess et al. [26] performed BAT biopsies from the cervical area in humans and they found that this tissue was predominantly beige. Furthermore, some studies postulated that maybe humans do not have classical BAT as mice do [25]. These studies hypothesized that supraclavicular human BAT is predominantly brite/beige adipose tissue enrolled by WAT. Moreover, the observed differences in BAT radiodensity between the cervical and thoracic areas could be based on the fact that BAT of the cervical area seems to be predominantly beige[27]. Nevertheless, the BAT within the thoracic area could be wrapped by WAT, as other studies have postulated [25,28]. This could explain the higher proportion of less dense tissue observed in comparison to cervical BAT. The possible fact that BAT is

wrapped by WAT in the thoracic area might also explain why overweight and obese individuals have a higher proportion of adipose tissue (including BAT and WAT) and slightly higher levels of BAT (only when PET-scans were excluded) in lower ranges of radiodensity compared with underweight and normal weight individuals. It should be noted that the metabolic activity of BAT upon a cold exposure was not different between categories of BMI.

One of the possible explanations of the differences in radiodensity by weight status is that adiposity is positively associated with oedema [29,30]. Edema or dropsy is a condition characterized by an excess of water fluid collecting in the cavities of tissue of the body [30]. Thus, it might be possible that the differences in fat adipose tissue radiodensity across weight-status categories could be explained by the fact that the adipocytes located in the supraclavicular and neck areas have an excess of water fluids.

We observed that women had less dense adipose tissues, as well as less dense BAT in comparison to men independently of the BMI. This could mean that these differences might be a sex-feature more than a weight status-feature. Moreover, we observed that women had a higher 18F-FDG uptake by BAT in the whole range of HU. Therefore, to have higher density of BAT does not necessary mean higher 18F-FDG uptake by this tissue, as in the present study we found the opposite in women. Further studies are needed to characterize the differences in density of adipose tissues between females and males.

Kinetics of BAT radiodensity

To date, only cold acclimation studies [31–34] or interventions with mirabegron (β3 adrenergic receptor agonist) [35,36] seem to activate and recruit human BAT in terms of 18F-FDG uptake. Some interventions such as exercise modified the body composition of the individuals (losing fat mass and gaining lean mass) [37]. In the light of these results, it will be interesting to also study whether the interventions used to activate BAT have an effect on BAT radiodensity. That is, if a participant would modify his/her body composition after the intervention, probably their BAT radiodensity could be modified going from less to denser ranges of HU, based on the body-weight differences that we have seen in the present study. A reduction in BAT radiodensity in combination with the same levels of activity (18F-FDG uptake) might be beneficial for individual's health, although the 18F-FDG uptake by BAT does not change. Since the mean value of HU seems to be negatively associated with indirect markers of adiposity [14], it could be beneficial for humans to perform some interventions orientated to modify BAT radiodensity more than to increase the 18F-FDG uptake, because the impact in humans health could be higher.

The results of the present work should be interpreted with caution because we only included the upper part of the body where BAT is mainly located [18], thus including other BAT depots (such as pararenal depots) could modify the results. It is well known that brown adipocytes not only consume glucose, but also (triglyceride-derived) fatty acids [38]. Since our study is based on an analogue of glucose, this is an important limitation. Therefore, it will be helpful to study the BAT radio-density with other tracers such as 18F-fluoro-6-thia-heptadecanoic acid or 11C-acetate. We do not know whether it can be applied to older or unhealthy people.

CONCLUSIONS

We showed that SUV thresholds determines BAT quantification more than HU thresholds. We showed that it is important to include the range from -10 to -50 HU because its contain a huge amount of the total BAT volume (43.2%), which represented 41.4% of the total BAT metabolic activity in our study. We observed that BAT volume radio-density was not different between categories of BMI but was different between sexes. All future human studies using static 18F-FDG-PET/CT scans should include the range from -10 to -50 HU, which mainly represent BAT located in the cervical area.

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PART 3

Brown adipose tissue and thermoregulation

Concurrent validity of supraclavicular skin temperature measured by iButtons and infrared thermography as a surrogate maker of brown adipose tissue

CHAPTER 8

BACKGROUND

Brown adipose tissue (BAT) is a thermogenic tissue able to release heat through the action of the uncoupling protein 1, an inner mitochondrial protein which uncouples oxidation from ATP synthesis [1]. In 2009, a set of studies confirmed the presence of metabolically active BAT in adults [2–4]. Nowadays, human BAT is being studied as a possible tool to combat obesity and metabolic-related diseases [5]. The main benefits of activating BAT is that it could play an important role in adaptive thermogenesis [6,7], as an endocrine organ [8] or in the thermoregulatory system [9].

The most used technique to activate human BAT consists in exposing participants to cold temperatures for approximately 2 hours [10]. Normally, after the first hour, an 18F-Fluorodeoxyglucose (18F-FDG) dose is injected, and, after the second hour, a positron emission tomography with computed tomography scan is performed (PET/CT) to assess BAT volume and activity [11,12]. However, this technique presents several limitations which hamper the understanding of this tissue in humans [11]. Consequently, there is a need to develop alternative and valid techniques which could solve these limitations.

Supraclavicular skin temperature has been previously proposed as a surrogate marker of human BAT [13–16]. A recent study showed that the temperature of the BAT depots located at the supraclavicular site can be measured by magnetic resonance spectroscopy (MRS) [17], whereas the supraclavicular skin temperature is normally measured with iButtons (small devices attached to the skin) [14] or by infrared thermography images (IRT) [18]. Two studies reported that supraclavicular skin temperature measured with iButtons is an indirect marker of BAT activity [14,15]. Both studies presented controversial data despite having used a similar sample and study design. On the other hand, the validity of supraclavicular skin temperature measured by IRT was examined by Law et al. [16], who showed that it could be used as a surrogate marker of BAT activity. Nonetheless, this study presents several limitations: (i) the small sample size (n=8) and (ii) the fact that the 18F-FDG-PET/CT scan and the IRT pictures were performed on different days and with different cooling protocol durations. Moreover, Sarasniemi et al. [19] showed that supraclavicular skin temperature measured by IRT positively correlates with the temperature of the BAT depots measured by MRS in lean adults, but negatively in obese adults. They observed that the fat layer (thickness) of the supraclavicular zone could play an important role, which concurs with other studies [20]. It is important to note that these studies were performed in thermoneutral conditions, hence we do not know whether the supraclavicular fat layer could also play a role during cold exposures. Sarasniemi et al. [19] highlighted the need to validate IRT against a method which truly measures the skin temperature. The supraclavicular fossa includes [21] a set of vessels, lymph nodes, and fat and skeletal muscles, which have their own temperature, and therefore it may be involved in the supraclavicular skin temperature. In addition, skeletal muscle seems to be the main tissue involved in the cold-induced thermogenesis [22]. Therefore, supraclavicular skin temperature may not represent only BAT thermogenic activity.

The present work has two aims: (i) to study the concurrent validity of skin measured with iButtons and IRT and (ii) to study the association of supraclavicular skin temperature measured with iButtons and IRT with BAT volume and activity quantified by 18F-FDG-PET/CT scan following the current recommendations [12].

MATERIAL AND METHODS

Participants

A total of 12 young adults (10 women) aged 18-25 years old participated in the present study. The measurements were conducted in May 2017 after the exercise intervention of the ACTIBATE study in a subsample of participants in Granada (Spain)[23]. The participants were healthy, non-smokers, with no family history of type 2 diabetes, they did not take any medication that could affect

thermoregulation, and were not pregnant. They signed a written informed consent before their enrolment. The study protocols were approved by the Human Research Ethics Committee of the University of Granada (n° 924) and Servicio Andaluz de Salud (Centro de Granada, CEI-Granada) and were performed according to the Declaration of Helsinki (Fortaleza, Brazil, October 2013).

Design

The participants were assessed on two study days with a 48-72h-separation (Figure 1). The participants were asked to attend the research center by bus or by car, after a minimum of 6h in fasting condition on both days. They were given instructions for the study days: they were requested to sleep as usual, to avoid moderate or vigorous physical activities for 24 and 48h, respectively, and to avoid any alcoholic or stimulant drink (at least 6h), or the use of body lotions or drugs (at least 24h) affecting the peripheral circulation. They were encouraged to be hydrated by drinking at least 1L before starting the measurements.

Procedures

Shivering threshold test

An extended description of the shivering threshold test can be found elsewhere [6,24]. On the first visit, the participants were required to wear standardized clothes (sandals, T-shirt, and shorts; clothing insulation value: 0.20), and they entered the warm room (~22.5°C), where they remained seated for 30 min. They were informed about the protocol and were asked to stay as still and calm as possible, avoiding getting up, rubbing, or covering their bodies. Furthermore, women were asked to tie their hair up. After that, they entered a cold room (~19.5°C) and remained seated for 15 min. At this point, a temperature-controlled water perfused cooling vest (Polar Products Inc., Ohio, USA) was placed and correctly adjusted on the participants' torsos, covering the anterior and posterior part of their trunk. Initially, water vest temperature was set at 16.6°C and reductions of 0.6-2.2°C were made every 10 min until a water temperature of 5.5°C was reached. If the participants or the researchers reported or perceived no shivering, additional 0.6°C reductions were performed every 15 min until either shivering or reaching a water temperature of 3.8°C. Those participants who did not shiver by this time remained in the cold room for 45 extra minutes.

Personalized cooling protocol

After 48-72 hours, we performed a personalized cooling protocol before the BAT quantification [24]. Upon arrival, the participants confirmed that the previous considerations were fulfilled. They were asked to urinate and to wear the same standardized clothes that they wore at the shivering threshold test. Women were asked to tie their hair up. Then, the participants entered the warm room (~22.5°C) and remained seated for 30 min. Subsequently, they entered the cold room (~19.5°C) where participants put on the same cooling vests with an initial water temperature of ~ 4°C above the personalized shivering threshold temperature (3.8 °C for those participants who did not shiver in the shivering threshold test). They were asked to stay in a sitting position during 120 min. If shivering was reported or detected, the water temperature was immediately increased by 1°C and a bathrobe was worn for 2 min. After 60 min of cold exposure, 185 MBq of 18F-FDG (~2.78 MBq/kg) were intravenously injected and the water temperature of the vest was increased by 1°C for the second 60-minute period. At this point, the PET/CT scan was conducted. A peak kilovoltage of 120 was applied for the CT acquisition, and for PET obtainment 2 bed positions (from atlas vertebra to mid-chest) were scanned, with an exposure time of 6 min per bed position (a total of 12 min).

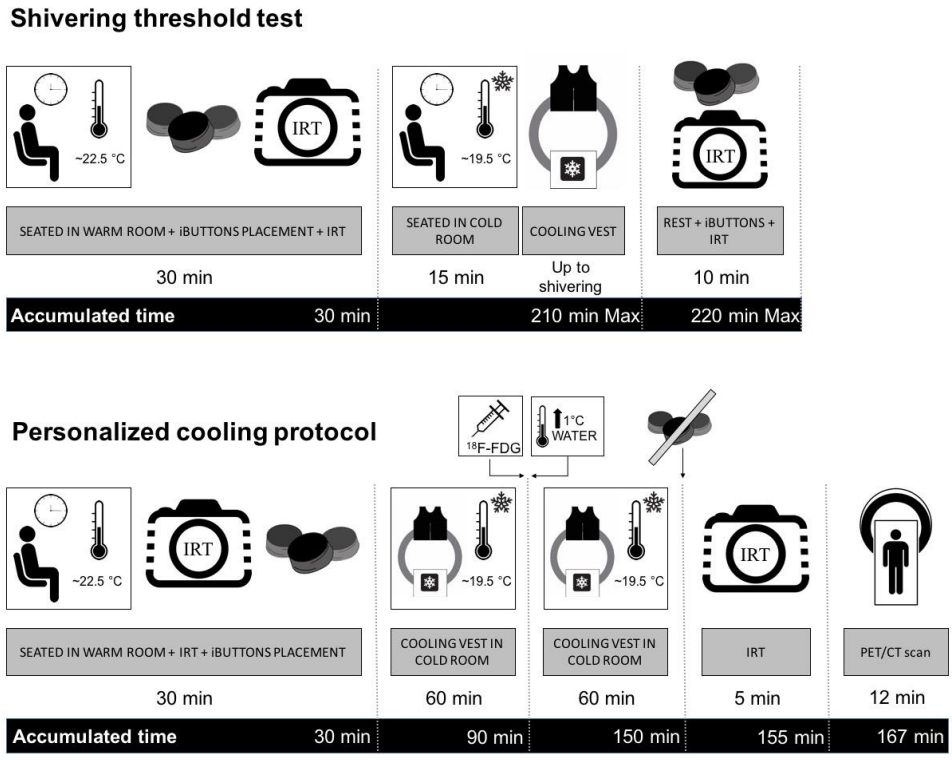

Figure 1. Design of studies of both study days.

Skin temperature measurements: iButtons

A total of 5 iButtons (DS-1922 L, Thermochron; resolution: 0.0625°C; Maxim, Dallas, USA) [25] were used to measure skin temperature in both protocols (i.e. shivering threshold test and the personalized cooling protocol). The iButton placement was performed at the beginning of both study days, when the participants were in the warm room [25,26]. For the current study, we analyzed data from the iButtons placed on the supraclavicular and sternal regions, forearm, index fingertip, and posterior part of the neck. One-minute intervals were selected as the recording frequency. The iButton data was analyzed using the Temperatus software (http://profith.ugr.es/temperatus?lang=en).
The validity and reliability of iButtons in the assessment of skin temperature in humans have been previously reported [27,28]. The supraclavicular, sternal, and forearm's temperatures were used in these analyses in order to include central, reference, and peripheral body regions.

Skin temperature measurements: IRT

For IRT acquisition, the participants went to a separate room where they took their T-shirts off and sat down for 5 min in a thermoneutral ambient for acclimatization (24.3 ± 1.6°C). Women were asked to move the straps of their sports bra aside, as well as to tie their hair up to make the supraclavicular area visible. IRT acquisition was measured before and after the shivering threshold test and before and after the personalized cooling protocol, just before the 18F-FDG-PET/CT scan. For each measurement, we took 4 thermal images using a FLIR E60 thermal imaging camera (FLIR Systems Inc., Wilsonville, USA) with thermal resolution set at 320 x 240 pixels. The first image was taken to an aluminum foil phantom (1 meter away) to obtain a measurement of the reflected

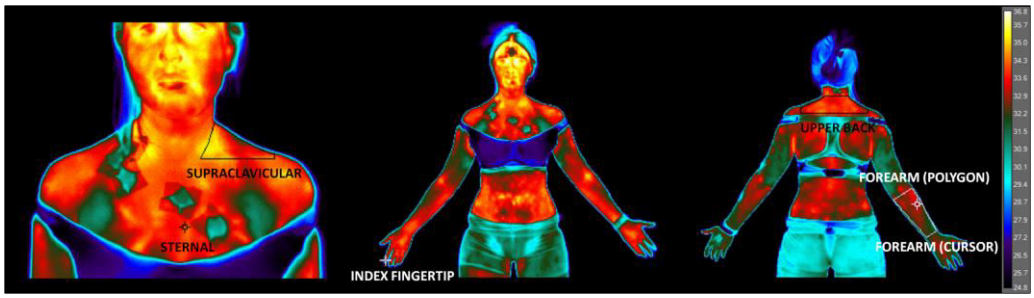

Figure 2. Anatomical location of the selected Regions of Interest (ROIs): supraclavicular, sternal, index fingertip, forearm (using both polygon and cursor ROIs), and upper back. The temperature scale (°C) is depicted on the right side of the figure. The thermal images were analyzed using the FLIR Research IR Software version 4.40.6.24 for Windows (FLIR Systems Inc., North Billerica, MA, USA).

temperature for each set of images, and the real time ambient temperature was registered for each picture. For the second thermal image, the participants remained seated in an upright position, with their arms relaxed on both sides of their legs. After a calculation of optimal distances, the camera was placed 1 meter from the midpoint of the chair for these images. For the third image, we removed the chair and we placed the camera 3 meters from the participant, who remained in an anatomical position facing the camera. For the last image, the participants turned 180° maintaining the same position and with their backs facing the camera, which was placed 3 meters away. Two thermal images were taken and the clearest one was retained for analyses. All the images were taken perpendicularly with the 5 body regions selected, which were the same regions where we had placed the iButtons. The pictures of the IRT were taken with the iButtons attached to the body, only in the shivering threshold test. We manually drew a region of interest (ROI) for each body region (Figure 2). We tried to draw these ROIs as close as possible to the iButtons' locations but in the opposite side of the body to prevent the iButton temperature from contaminating the skin temperature, except for the sternal and upper back regions, where we drew the ROI just below the iButtons. All ROIs were performed using the FLIR ResearchIR Max software version 4.40.6.24 for Windows (FLIR Systems Inc., North Billerica, MA, USA). The supraclavicular and sternal ROIs were derived from the second image, the index fingertip ROI from the third image, and the forearm and upper back ROIs from the fourth image. All analyses were adjusted by atmospheric temperature and relative humidity, which were measured using a FLIR MR77 moisture meter (FLIR System Inc., Wilsonville, USA) at the beginning of each set of thermal images. Furthermore, the reflected temperature was obtained by placing a rounded ROI on the aluminum foil phantom of the first thermal image and retaining the mean value (°C) for adjustments. For all thermal images, emissivity was set at 0.98. Minimum, maximum, and mean values of each ROI were retained as variables.

PET/CT analyses

The Beth Israel plugin for FIJI software [29] was used to analyze the PET/CT images. ROIs were semi-automatically drawn from the atlas vertebra to the thoracic vertebra 4 using a 3D-Axial technique [30]. Our protocol has recently shown high inter-observer reliability [31]. The standardized uptake value (SUV) was calculated as follows: 18F-FDG uptake (kBq/mL)/ (injected dose [kBq]/patient weight [g]). BAT volume, SUVmean, and SUVpeak were defined according to BARCIST 1.0 criteria [i.e. SUV threshold individualized to lean body mass in combination of Hounsfield Units (HU) range from -10 to -190] [12]. BAT volume (ml) was calculated as the sum of the volumes defined as BAT in each ROI. SUVmean (g/ml) was obtained from the weighted average of the SUVmean of each ROI. Finally, SUVpeak (g/ml) was the highest average SUV in a 1 ml spherical volume. We

drew a single ROI from 1 slice in supraspinatus,paracervical,sternocleidomastoid, longus colli, trapezius, parathoracic, subscapular, deltoid, pectoralis major, scalene, and triceps brachii muscles from both left and right side of the body [32,33]. An average of both sides including all skeletal muscles was performed in order to obtain a single representative value of the skeletal muscle 18F-FDG uptake of the upper part of the body. Moreover, we performed different skeletal muscle groupings [32].

Body composition

We measured the participants' weight and height barefoot and wearing the standardized clothes using a SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Body mass index (BMI) was calculated as weight/height squared (kg/m2). Additional body composition measurements (i.e. lean mass and fat mass) were taken by Dual Energy X-ray Absorptiometry scan (Discovery Wi, Hologic, Inc., Bedford, MA, USA). Lean mass index and fat mass index were calculated as lean mass/height squared and fat mass/height squared (kg/m2), respectively.

Statistical analysis

The descriptive characteristics of the study sample are shown as mean ± standard deviation and as percentage when stated in Table 1. To study the concurrent validity between the iButtons and IRT, we performed paired T-tests comparing both devices' outputs measured in the shivering threshold test. We studied which outcome of skin temperature measured by IRT (i.e. minimum, maximum, or mean) in different body regions and in different conditions (i.e. before and after cold exposure) showed a more similar measurement to that obtained from the iButtons. The iButton output used for these comparisons was obtained at the same time as the thermal image was taken. In order to study the grade of agreement between iButtons and the mean IRT measurement we applied the Bland-Altman method [34]. Later, we performed one-sample t-tests to analyze whether the differences between both devices were statistically significantly different from 0. Additional one-sample t-tests were performed using the absolute difference between the methods to avoid potential balances between positive and negative values, which could lead to wrong conclusions.

To study the association between supraclavicular skin temperature measured with iButtons and IRT in the personalized cooling protocol and human BAT volume and activity, we conducted simple linear regression analyses. We used additional simple linear regression analyses to observe whether sternal or supraclavicular skin temperature or the difference between supraclavicular minus sternal r were able to predict human BAT as previously suggested [16]. These analyses were repeated introducing the 18F-FDG uptake by skeletal muscles in the model instead of BAT.

We performed one-way analysis of variance (ANOVA) with repeated measures to study the effect of cold on skin temperature parameters for both devices.

RESULTS

Table 1 shows the characteristics of the participants of this study. All subjects participated on both study days. However, we could not obtain the iButtons and IRT data of one participant during the personalized cooling protocol, and the same occurred with another participant during the shivering threshold test. This is the main reason why we have the same sample size but different

Table 1. Participant's characteristics (n=11).

	Shivering threshold test					Personalized cooling protocol				
	Mean		SD	Min	Max	Mean		SD	Min	Max
Sex (% male)	(n=2) 18.2%					(n=2) 18.2%				
Age (years)	21.9	±	2.2	18.3	25.8	22.2	±	2.3	18.3	25.8
Body mass index (kg/m2)	23.5	±	4.8	18.4	34.8	23.9	±	4.7	18.4	34.8
Lean mass index (kg/m2)	14.6	±	2.6	11.5	21.3	14.7	±	2.5	11.5	21.3
Fat mass (%)	32.7	±	6.9	16.6	40.7	32.7	±	7.3	16.6	40.7
Fat mass index (kg/m2)	7.7	±	2.7	3.0	11.8	7.9	±	2.7	3.0	11.8

Data are presented as mean ± standard deviation (SD) or percentage when stated

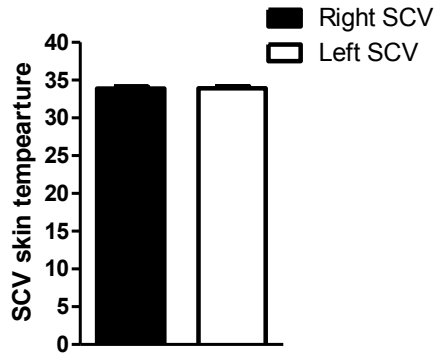

Figure 3. Differences between supraclavicular skin temperatures (SCV) measured in the right and left fossa with infrared thermography (IRT). The data represents the average of skin temperature measured by IRT at the end of the personalized cooling protocol.

descriptive data. Of note is that there was a physiological outlier (BMI=35 kg/m2). We performed sensitivity analyses excluding this participant from the analyses and the results persisted (data not shown). Moreover, we observed that supraclavicular skin temperature in the same cohort measured by IRT from the right and left side was not different after the personalized cooling protocol (Fig. 3).

Concurrent validity

Table 2 shows the differences between the average of the iButton measurement of skin temperature in the different body regions and the minimum, maximum, and mean measurement of skin temperature by IRT. We observed that there were significant differences between the iButtons and the minimum measurement of IRT in 4 out 5 body regions (supraclavicular, sternal, forearm, and upper back) before and after the shivering threshold test (all P<0.05). We found similar differences between the iButtons and the maximum measurement of IRT in the same body regions (all P<0.05). However, regarding the differences between the iButtons and the mean measurement of IRT, the only difference found was in the sternal region (P<0.05), while the supraclavicular, forearm, index fingertip, and upper back regions showed no differences

Table 2. Differences between skin temperatures of the selected body regions measured with iButtons and infrared thermography (IRT) before and after the shivering threshold test.

				Before cold							
	n	iButtons (ºC)		n	IRT minimum (ºC)		IRT maximum (ºC)		IRT mean (ºC)	
Body region	iButtons	mean	SD	IRT	mean	SD	mean	SD	mean	SD
Supraclavicular	11	34.40	0.78	11	32.40***	1.38	35.47***	0.64	34.55	0.71
Sternal	11	33.38	1.08	10	34.16*	0.89	34.32**	0.84	34.23*	0.88
Forearm	9	33.20	0.95	11	30.44**	1.32	33.41	0.65	32.54	0.73
Index fingertip	11	32.16	1.74	11	31.21	1.79	31.21	1.79	31.21	1.79
Upper back	11	34.01	0.84	11	31.12**	2.15	34.77*	1.29	33.71	1.17
				After cold						
	n	iButtons (ºC)		n	IRT minimum (ºC)		IRT maximum (ºC)		IRT mean (ºC)	
Body region	iButtons	mean	SD	IRT	mean	SD	mean	SD	mean	SD
Supraclavicular	10	34.56	0.55	10	31.41***	1.00	35.50***	0.56	34.29	0.83
Sternal	10	33.01	1.29	10	33.61*	1.04	33.76*	1.01	33.68*	1.00
Forearm	8	29.87	1.23	10	27.28**	1.32	31.43**	1.01	29.98	1.08
Index fingertip	10	22.95	1.36	9	23.73	0.84	23.73	0.84	23.73	0.84
Upper back	10	32.91	1.44	10	29.51***	2.24	34.60***	1.08	32.98	1.31

* p<0.05; ** p<0.01; *** p<0.001 for differences between iButtons and IRT measurements. Significant differences are highlighted in bold in the IRT data.

(all P>0.05). These results were similar before and after the shivering threshold test.

Based on the results displayed in Table 2, we selected the measurement of iButtons and the mean measurement of the IRT to perform the Bland-Altman plots (Fig. 4). The systematic error between the iButtons and the mean measurement of IRT was not significantly different from 0 for the supraclavicular region before and after the shivering threshold test (mean differences ranged from -0.15 to 0.27°C, all P>0.05, Fig. 4).

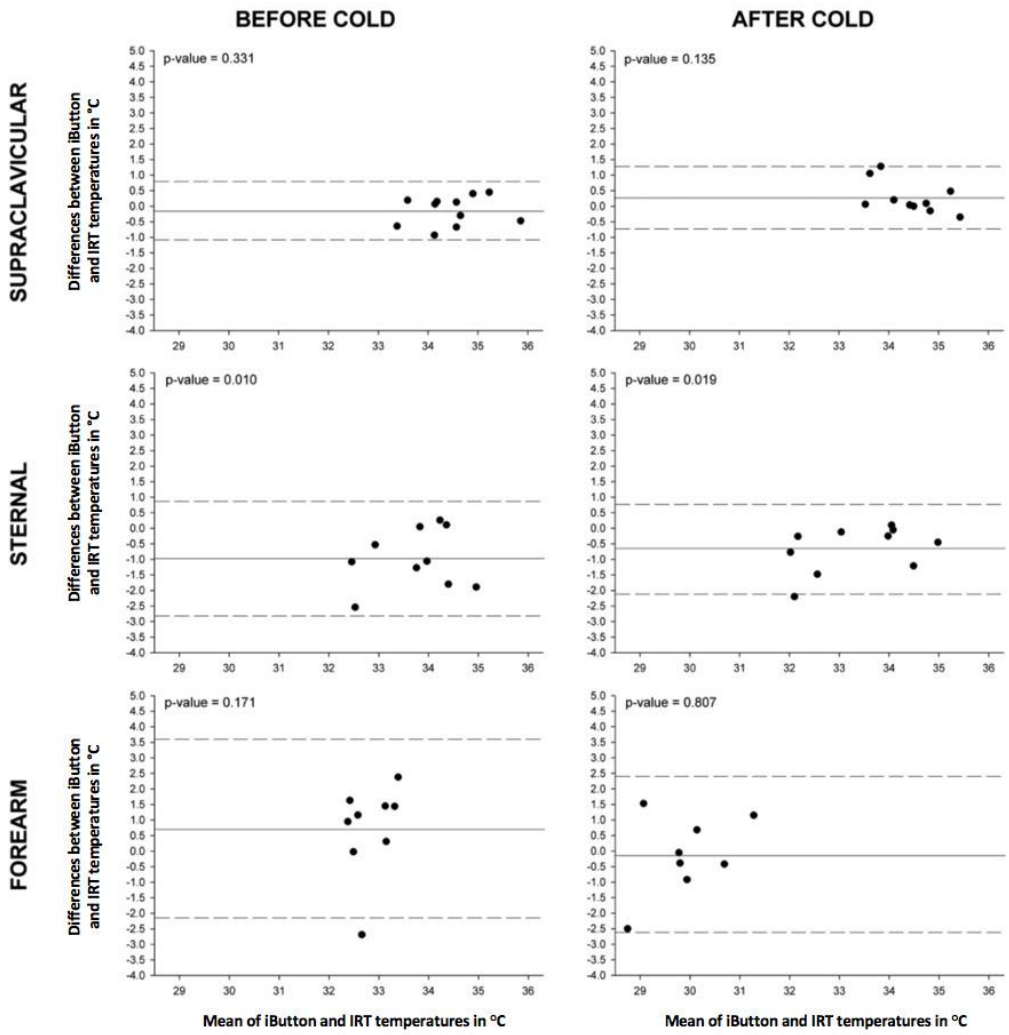

Figure 4. Bland-Altman plots of the supraclavicular, sternal, and forearm mean skin temperatures measured with infrared thermography (IRT) [region of interest (ROI's) mean temperature] and iButtons during the shivering threshold test. The central lines represent the mean difference between the iButton and IRT measurements; the dashed lines represent the upper and lower 95% confidence intervals, respectively. The P values represent whether the mean differences were significantly different from 0.

For the sternal region, the systematic error was statistically significantly different from 0 ($P<0.05$) before and after cold (mean differences ranged from -0.67 to -0.97°C). Regarding the forearm, the systematic errors for the two different conditions did not differ from 0 (mean differences ranged from -0.11 to 0.74°C, All $P>0.05$). We transformed the value of the differences between the iButton and IRT to absolute values, and we studied whether these outcomes were statistically different from 0 (Fig. 5). We observed that the differences between instruments in all the regions and conditions (before and after cold exposure) were different from 0.

Association between supraclavicular skin temperature with 18F-FDG uptake by BAT

Supraclavicular skin temperature measured with the iButtons and the mean of measurement of IRT were not associated with any BAT-related outcome (all $P\geq0.165$, Fig. 6A and B for iButton and IRT). However,

we found a negative association of supraclavicular skin temperature measured by IRT with BAT volume (r=-0.764; P=0.006), but not with supraclavicular skin temperature measured with the iButtons (r=-0.546; P=0.082). These results persisted when we included fat mass percentage as a covariate (data not shown). We selected the sternal region as a reference location because this region did not change upon the cold exposure whereas other regions did (Fig. 8). In addition, we did not observe any association between sternal skin temperature and BAT-related outcomes (Fig. 6C and D, for the iButton and IRT, respectively) (Fig 6). All the analyses were performed with the data of skin temperature after the personalized cooling protocol. When we analyzed the association of the difference between supraclavicular minus the sternal skin temperatures and BAT-related outcomes, we did not find any significant association (All P≥0.061, Fig. 7A and B). When we studied these associations before cooling, the results persisted (data not shown). Moreover, we did not find any association between the differences in supraclavicular skin temperature (after-cold minus the measurement before-cold conditions) and BAT-related outcomes with both devices (data not shown). Furthermore, we repeated the associations with BAT-related outcomes obtained only in the supraclavicular fossa and the results persisted (data not shown). Finally, we repeated all the analyses between supraclavicular skin temperature with both devices and 18F-FDG uptake by skeletal muscles and we did not find any association (data not shown).

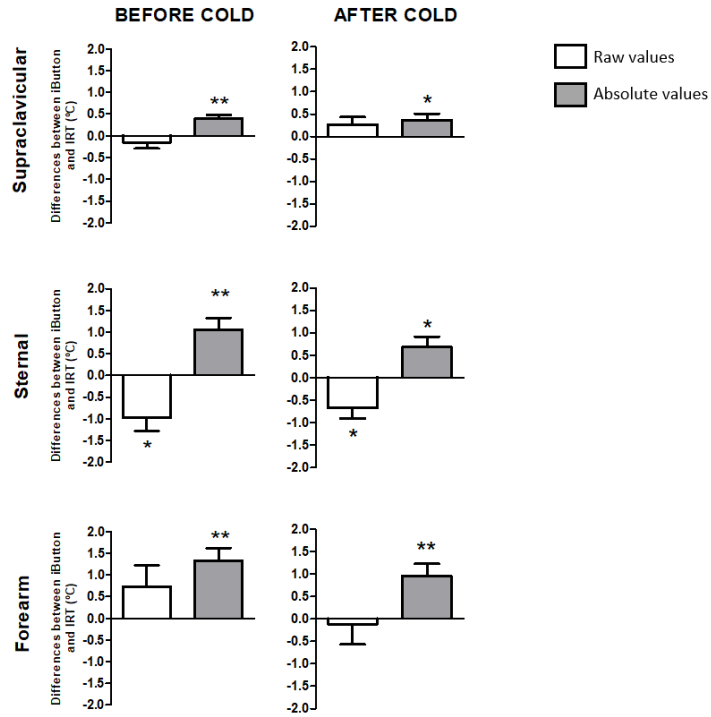

Figure 5. Histograms show the values of skin temperature measured with iButtons minus the average of mean IRT in the supraclavicular, sternal, and forearm regions, before and after the cooling protocol. The white boxes depict raw values of the differences, whereas grey boxes show the absolute values. * Symbol means P<0.05 in the one sample t-test from 0; ** means P<0.01 in the one sample t-test from 0.

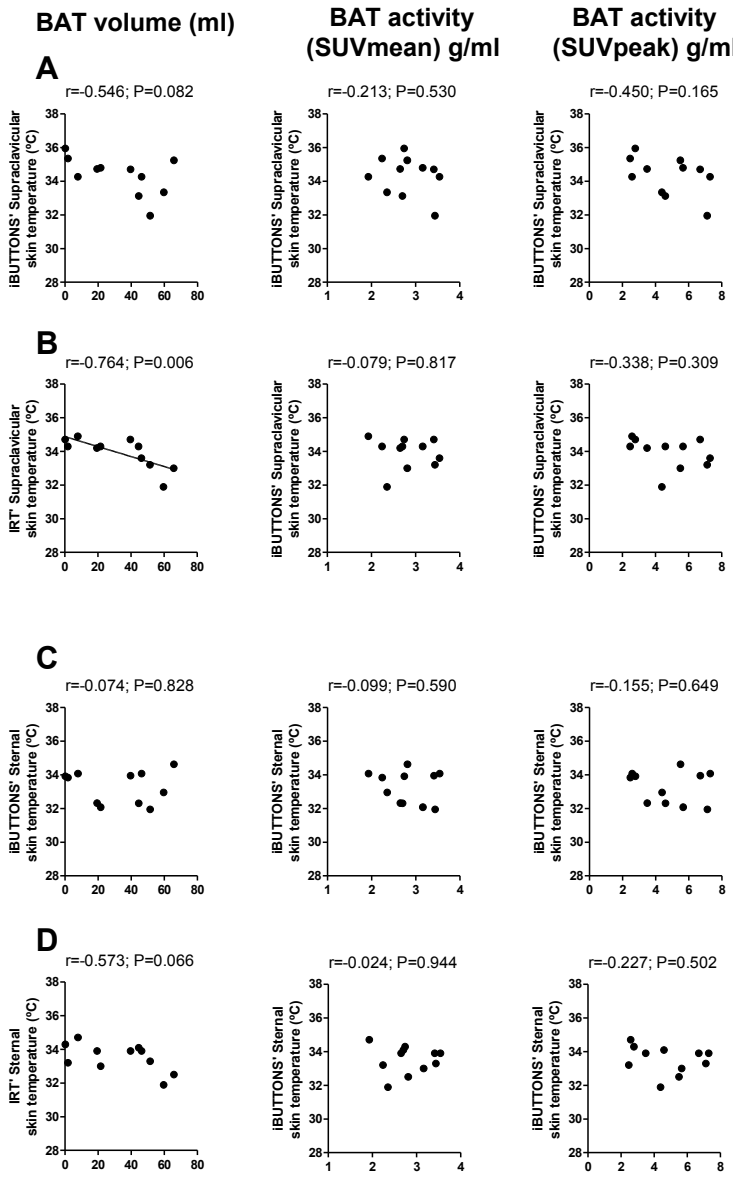

Figure 6. Associations between supraclavicular skin temperature at the end of the personalized cooling protocol measured with iButtons (A) and infrared thermography (IRT) (B), and brown adipose tissue (BAT) volume and activity (SUV mean and SUVpeak). Panels C and D show the same association but with sternal instead of supraclavicular skin temperatures.

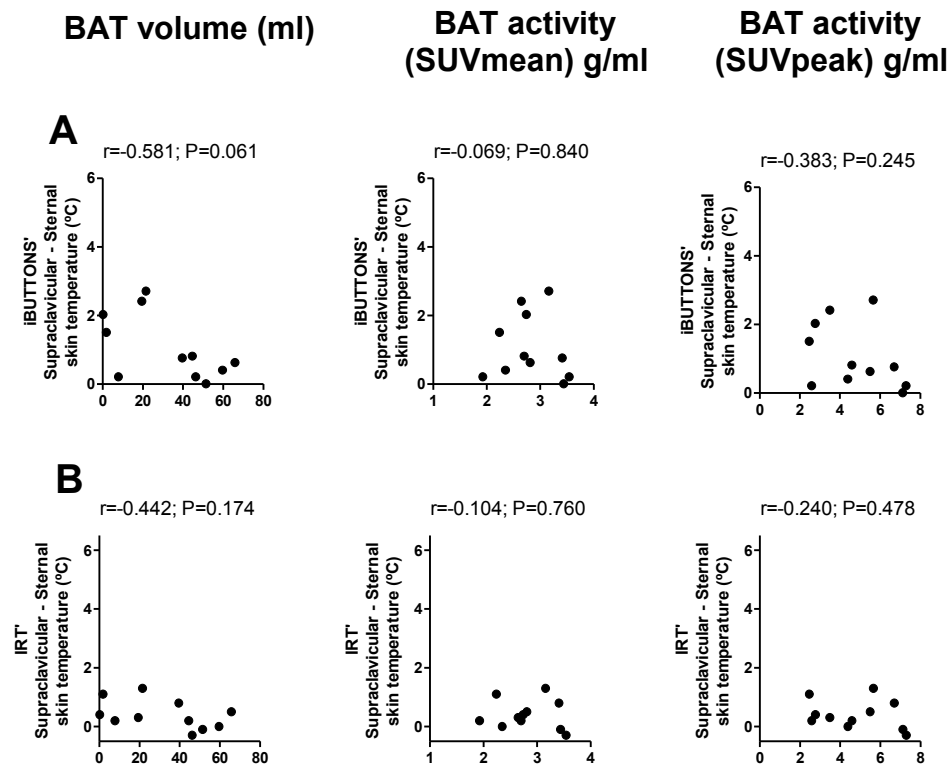

Figure 7. Associations between supraclavicular skin temperature relative to sternal skin temperature at the end of the personalized cooling protocol and brown adipose tissue (BAT) volume and activity measured with iButtons (A) and by infrared thermography (IRT) (B).

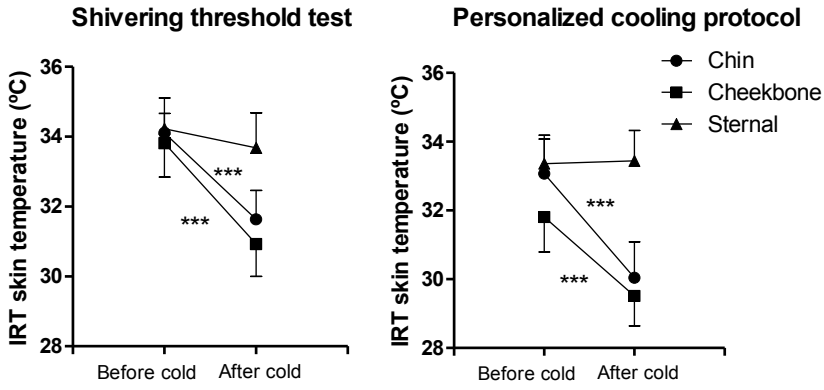

Figure 8. Differences in the chin, cheekbone, and sternal skin temperatures (as the reference locations) in the cooling protocols measured by infrared thermography (IRT). *** Symbol means significant differences in relation to the baseline (P<0.001).

DISCUSSION

The present study shows that the skin temperature measured with iButtons and IRT is not comparable. Moreover, there is no association between supraclavicular skin temperature measured with iButtons or the mean measurement of IRT and BAT activity (SUVmean or SUVpeak). There was, however, a negative association between the supraclavicular skin temperatures measured by IRT and BAT volume.

Concurrent validity

The interest in the thermoregulatory response to different stimuli (exercise, meal intake, or cold) has increased given the facility and the relatively low cost of implementing these measurements [11]. iButtons are small and inexpensive thermometers used in different fields. However, their validity is questioned, since these devices are able to measure by both sides [35,36], although this issue has not been addressed yet. On the other hand, IRT creates an image by converting radiant heat energy into a signal [11,19]. In light of the present findings, we found that iButtons and IRT outcomes are not comparable. Currently, we do not know which device is better for the measurement of skin temperature, because these devices have not been compared with a device that truly measures skin temperature (for instance, mercury's thermometer or thermocouples), which is of pressing need in the field.

Supraclavicular skin temperature does not seem to be a valid tool to quantify human BAT in young adults

To date, only 2 studies have validated the use of supraclavicular skin temperature (measured with iButtons) as a surrogate marker of BAT activity measured with the 18F-FDG-PET/CT scan. Both studies were conducted in young lean and healthy adults, but their results were controversial. Boon et al. [14] found that supraclavicular skin temperature was associated with BAT activity (i.e. SUVmean and SUVmax) at the end of the cold exposure, whereas van der Lans et al. [15] found that the difference between supraclavicular skin temperature after-cold minus before cooling was associated with BAT activity (i.e. SUVmean and SUVmax). We found, however, no association between supraclavicular skin temperature measured with iButtons or by IRT and BAT activity. Law et al. [37] studied the validity of supraclavicular skin temperature measured by IRT with BAT. They performed the IRT and dynamic 18F-FDG-PET/CT quantification on separate days and, more importantly, the thermal images were performed after 10 minutes of cold exposure, whereas the metabolic rate of glucose [MR(gluc)] by BAT was obtained after 60 minutes. They reported that when the supraclavicular skin temperature was relativized to sternal skin temperature, it was positively correlated with MR(gluc) by BAT in 8 lean male individuals, yet the absolute values of supraclavicular skin temperature were not associated. They justified performing the IRT pictures after 10 minutes of cold exposure, because BAT shows its higher peak of activity at this point. However, this assumption has not been demonstrated with nuclear medicine techniques. In the present work, we quantified supraclavicular skin temperature before and after 2 hours of a personalized cooling protocol, we performed the IRT measurements just before performing the BAT quantification, and we applied the latest recommendation for static 18F-FDG-PET/CT scan [12]. We also demonstrated that the sternal skin temperature, compared to chin and cheekbone skin temperature, is a real reference location upon a cold exposure (Figure 8). However, we failed to replicate the results obtained by Law et al. [37]. We included a larger sample size and both men and women in comparison to Law et al. [37] [12 individuals (men and women) vs. 8 men]. In addition, our sample was less homogeneous in terms of body composition in comparison to Law et al. [37].

Furthermore, Sarasniemi et al. [19] recently observed that the supraclavicular skin temperature measured by IRT was

negatively and significantly associated with BAT-depot temperature located in the supraclavicular region (r=-0.83, P=0.042) only in obese participants. Similarly, we observed that supraclavicular skin temperature measured by IRT was negatively associated with BAT volume. We thought that our outlier according to BMI levels (35kg/m2) could be driving the association, but when we removed this participant the negative significant association remained. Saraniesmi et al. [19] discussed that the supraclavicular fat layer (which is positively related to BMI [20]) could be playing a cofounding role in the measurement of supraclavicular by IRT. Indeed, when they included the supraclavicular fat layer as a co-variate, the negative association disappeared. Of note is that, when we included the fat mass percentage or BMI the observed negative association persisted. A possible explanation could be that these outcomes are not representative of the supraclavicular fat layer.

Moreover, they performed the complete experiment in thermoneutral conditions, whereas we performed the experiment after a personalized cold exposure, and we found the same direction of association only in BAT volume. We cannot discard that the supraclavicular fat layer played an important role in our study because our sample was comprised of mainly normal weight individuals. Therefore, future studies addressing this issue are warranted.

Blondin et al. [32] and U Din et al. [22] showed that skeletal muscles of the neck are highly involved in the cold-induced thermogenesis, which are also presented in the supraclavicular fossa [21]. In line with this hypothesis, we did not find any association with 18F-FDG uptake by skeletal muscles. In addition, supraclavicular depots are highly irrigated by vessels and lymph nodes placed around the neck, which may affect their temperature [21]. The instruments currently used in this field are not able to distinguish between tissues; therefore, supraclavicular skin temperature should be interpreted as a holistic measure of the reaction of all the tissues, which are located in the supraclavicular fossa to different environmental conditions.

We do not know whether these results would be replicated using a magnetic resonance imaging or a PET/CT scan with other tracers [11]. Moreover, these analyses should be replicated in older and obese individuals. For future studies, the measurement of the supraclavicular fat layer should be included as a covariate in order to account for its potential effect. Despite the fact that iButtons seem to be a valid and reliable tool for the assessment of skin temperature [27,28], these devices are able to measure temperature by both sides [35,36], which could be an important limitation in human physiology studies. Therefore, how this issue affects the measurement of the skin temperature should be addressed in the near future. As a result, more studies comparing the IRT measurements with alternative devices (i.e. mercury thermometers or thermocouples) are encouraged.

CONCLUSIONS

The present study shows that iButtons and the mean measurement of IRT are not comparable. Moreover, we did not observe any association between supraclavicular skin temperature measured with iButtons or by IRT and BAT activity, as well as with 18F-FDG uptake by skeletal muscle. We confirm a negative association between supraclavicular skin temperature measured by IRT and BAT volume, which could be partially explained by the supraclavicular fat layer. In light of these findings, supraclavicular skin temperature (regardless of the instrument used) is a valid instrument to quantify 18F-FDG uptake by BAT in young adults.

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Association of wrist and ambient temperature with cold-induced brown adipose tissue and skeletal muscle 18F-Fluorodeoxyglucose in young adults

CHAPTER 9

BACKGROUND

Almost a decade ago, it was discovered that humans present metabolically active brown adipose tissue (BAT) [1–4]. Since then, several studies have postulated this tissue as a possible target to face obesity, type 2 diabetes, and related comorbidities in humans [5]. The most effective physiological stimuli for the activation of BAT is cold exposure [6]. A consequence of cold exposure is the increase of energy expenditure, named cold-induced thermogenesis (CIT) [7], which seems to originate from the activation of BAT and/or skeletal muscle [8]. The individual contribution of each tissue to CIT remains however unclear.

Observational studies have consistently shown a negative association between outdoor temperature and 18F-FDG uptake by BAT (9–11). Indeed, 18F-FDG uptake by BAT (volume and activity) are higher at winter compared with warmer seasons [9]. Moreover, the abundance of beige adipocytes in the subcutaneous adipose tissue is also higher in winter [12]. Yoneshiro et al. [11] observed that CIT is also higher in winter, although this seasonal variation was only present in participants with detectable BAT. Since skeletal muscle also contribute to CIT [8], it is plausible that its cold-induced activity also follows this seasonal variation, although it has not been tested yet. Several studies have used cold-acclimation interventions to activate human BAT [13–15]. Some of these cold-acclimation studies [14,16] showed an up-regulation effect of cold air exposure on BAT and skeletal muscle 18F-FDG uptake. Therefore, these studies confirmed that the ambient temperature exposure before human BAT quantification is determinant.

It is noteworthy that the previous observational studies have used the outdoor ambient temperature (Outdoor-AT) measured by different meteorological national agencies of each region [17] as a representative value of ambient temperature exposures. Nevertheless, people spend most of their time indoors (roughly 90% of their time), especially in colder regions/seasons [18], making outdoor-AT inaccurate measures of true ambient exposure for each participant. Considering this, it is of interest to study the relation of the daily personal level of environmental temperature (Personal-ET) with 18F-FDG uptake by BAT and skeletal muscle activity. In addition, wrist temperature (WT) can be used as indirect measurement of distal body temperature in daily living conditions [17,19,20] and therefore, studying its relationship with BAT is of interest. Moreover, whereas the range of temperature at which BAT is activated is known [21], less information is available about the ranges of temperature to induce BAT inhibition

Thus, we analyzed the association of WT and Personal-ET measured between October and November 2016 in Granada (Southern Spain), with cold-induced 18F-FDG uptake by BAT and skeletal muscle activity in young adults. We also studied whether the time spent in a certain range of temperature is associated with the 18F-FDG uptake by BAT and skeletal muscles.

MATERIAL AND METHODS

A total of 96 young adults (n=65 women) aged 21.9 ± 2.3 years old participated in the study, of whom 21 were excluded from the analysis (n=10; 47.6% women) because they had less than 5 valid days of temperature records. A total of 75 participants (n=56; 74.6% women) were finally included in the study analysis (Table 1).

The participants were part of the ACTIBATE study [22], an exercise-based randomized controlled trial (ClinicalTrials.gov ID: NCT02365129). All participants were healthy, reported to be non-smokers and sedentary (less than 20 min physical activity on less than 3 days per week), had no family record of type 2 diabetes, and did not take any medication that could impact the cardiovascular or thermoregulatory responses to cold exposure. The cross-sectional measurements were performed in four waves of 24 participants each in Granada (Spain) from October 1st to November 27th, 2016. The study protocol and informed consent were designed in accordance with the last version of the Declaration of Helsinki.

The study was approved by the Ethics Committee on Human Research of the University of Granada (n° 924) and by the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada).

Wrist and ambient temperature measurements

All participants wore 2 iButtons (DS-1922 L, Thermochron; resolution: 0.0625°C; frequency: 10 min intervals; Maxim, Dallas, USA) during 7 days. One iButton was placed on the ventral side of the wrist of the non-dominant hand over the radial artery in a wrist band in order to quantify the distal body temperature in daily living conditions. To measure Personal-ET, another iButton was adhered to a plastic fob, which was always with the participant but never in direct contact with their bodies, neither under clothing [20]. They wore this iButton attached to keychain or in a bagpack or bag. The participants were instructed to wear the wrist iButton during day and night, even during the sleep-phases, and to take it off when bathing. During the sleep-phases, the Personal-ET sensor was placed on the bedside table. The participants registered the non-wear periods in a diary during the 7 days. The iButtons were programmed to start recording at 6.00 a.m. and to finish 7 days later at 12.00 a.m., the morning of the day when the 18F-FDG-PET/CT scan was performed. Furthermore, we downloaded the Outdoor-AT for Granada from the Spanish National Meteorological Agency, and we calculated the individual average of the Outdoor-AT during the 7 days previous to the 18F-FDG-PET/CT scan. The participants were measured in 4 different waves (in the same day we performed around 18 personalized cold exposures before 18F-FDG-PET/CT scan), and, therefore, the participants in the same wave shared the same Outdoor-AT. The average of Outdoor-AT of the 7 days previous to the 18F-FDG-PET/CT scan in the 4 waves were categorized into 9°C, 17.5°C, and 21.5°C (two waves shared exactly the same average: 17.5°C).

Based on the diary records, we excluded the non-wear periods as well as those participants with less than 5 days of valid registration. A valid day is defined as having registered at least 18 hours. Also, if WT was lower than 28°C or higher than 48°C, we excluded both measurements (WT and Personal-ET), since, physiologically, WT cannot measured these extreme temperatures during free daily living conditions [23,24]. All iButtons were programed and analyzed with the Temperatus® software (http://profith.ugr.es/temperatus?lang=en). WT and Personal-ET records were averaged, separately. We then calculated the average number of hours per day exposed to a certain temperature during the 7-day measurement period. For instance, we calculated the number of hours per day that the participants were exposed to 29.00°C-29.99°C. We performed these calculations with temperatures ranging from 11 to 32°C for Personal-ET and from 32° to 37°C for WT.

Personalized cooling protocol prior to 18F-FDG-PET/CT scan

The personalized cooling protocol has been explained in detail elsewhere [25]. Briefly, we gradually cooled down the water temperature of the cooling vest (Polar Products Inc., Ohio, USA) in a combination of mild-cold air room (~19.5°C) until shivering occurred. Shivering was determined visually by researchers and self-reported, and the lowest temperature at which the participants did not shiver was registered. After 48-72 hours, we exposed the participants directly to ~4°C above the temperature that caused the onset of shivering during 2 hours to induce maximum non-shivering thermogenesis [26]. A bolus of 18F-FDG was injected after 1 hour of cold exposure, after which the water temperature rose 1°C in order to avoid shivering. After 2 hours of cold exposure we performed the 2 bed positions from atlas vertebrae to thoracic vertebrae 6, including only the upper part of the body in the PET/CT scan (Siemens Biograph 16 PET/CT, Siemens, Germany).

Table 1. Descriptive characteristics of the participants (n=75).

	All (n=75)	1st wave (n=18)	2nd wave (n=19)	3rd wave (n=16)	4th wave (n=18)
Sex (women)	(n=56; 74.6%)	(n=15; 83.3%)	(n=12; 63.1%)	(n=11; 68.7%)	(n=14; 77.8%)
Age (years)	21.9 ± 2.3	21.9 ± 2.6	21.9 ± 2.1	22.2 ± 2.2	22.1 ± 2.3
Body mass index (kg/m²)	25.2 ± 4.8	25.4 ± 5.1	24.8 ± 4.1	26.8 ± 4.8	24.6 ± 5.6
Lean mass (kg)	41.3 ± 9.6	40.6 ± 9.1	43.0 ± 9.7	41.7 ± 9.6	40.5 ± 11.1
Fat mass (kg)	26.9 ± 9.5	28.2 ± 8.5	23.9 ± 8.8	30.5 ± 9.6	26.6 ± 10.9
Fat mass (%)	37.6 ± 7.0	39.3 ± 5.2	34.1 ± 8.5	40.6 ± 6.0	37.5 ± 6.9
BAT volume (ml)	69.0 ± 61.3	15.1 ± 29.2	61.1 ± 49.5	105.1 ± 53.4	100.3 ± 67.2
BAT activity (SUV_{mean} g/ml)	3.7 ± 1.9	2.0 ± 1.5	3.6 ± 1.7	4.8 ± 1.9	4.4 ± 1.6
BAT activity (SUV_{peak} g/ml)	11.0 ± 8.5	3.5 ± 3.8	9.9 ± 7.2	17.2 ± 9.8	14.0 ± 6.9
Wrist Temperature (ºC)	34.0 ± 0.7	34.4 ± 0.5	34.0 ± 0.5	33.5 ± 0.7	33.7 ± 0.8
Personal-ET (ºC)	22.8 ± 3.1	26.0 ± 1.2	23.8 ± 1.4	21.0 ± 2.4	20.6 ± 3.6
Outdoor-AT (ºC)	14.4 ± 5.4	21.5 ± 0.0	17.5 ± 0.0	9.3 ± 0.5	8.9 ± 0.0

Note: data are presented as mean and standard deviation, unless otherwise stated. BAT: brown adipose tissue, Outdoor-AT: Outdoor ambient temperature, Personal-ET: Personal level of environmental temperature, SUV: standardized uptake value.

BAT and muscle quantification

We quantified BAT volume and activity following the recent recommendations [27]. PET/CT images were analyzed using the Beth Israel plugin for FIJI [25], an example of the PET/CT images can be found elsewhere [25,28]. We applied a fixed range of Hounsfield units (HU, −190 to −10) and an individualized standardized uptake value (SUV) threshold [1.2/(lean body mass/body mass)]. We determined BAT volume, SUVmean (as a proxy of mean 18F-FDG uptake), BAT metabolic activity, SUVpeak (the average of the 3 highest pixels of 18F-FDG uptake located in less than 1 cm), and SUVmax (the highest 18F-FDG uptake in one pixel) [25] as well as the 18F-FDG uptake in a reference tissue (descending aorta). We quantified the 18F-FDG uptake (SUVpeak) of several skeletal muscles included between atlas vertebrae and thoracic vertebrae 4. We drew a single ROI from 1 slice in paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, suprasipinatous, subscapular, deltoid, pectoralis major, and triceps braquis muscles from both left and right side of the body [14,29]. An average of both sides including all skeletal muscles was performed in order to obtain a single representative value of the skeletal muscle 18F-FDG uptake of the upper part of the body. Moreover, we performed different skeletal muscle groupings [29].

Body composition

Body composition was measured by Dual Energy X-ray Absorptiometry scan (HOLOGIC, Discovery Wi). We measured the participants' weight and height barefoot and wearing the standard clothes (shorts and t-shirt) using a SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany).

Statistical analysis

The data are presented as mean ± standard deviation, unless otherwise stated. We conducted linear regression analyses to examine the association of WT or Personal-ET with 18F-FDG uptake by BAT (volume and activity) as well as with 18F-FDG uptake by skeletal muscle activity (dependent variables). In addition, we performed linear regression analyses to examine the association of WT and Personal-ET with the 18F-FDG uptake (SUVpeak) by the descending aorta (reference tissue) [27]. We

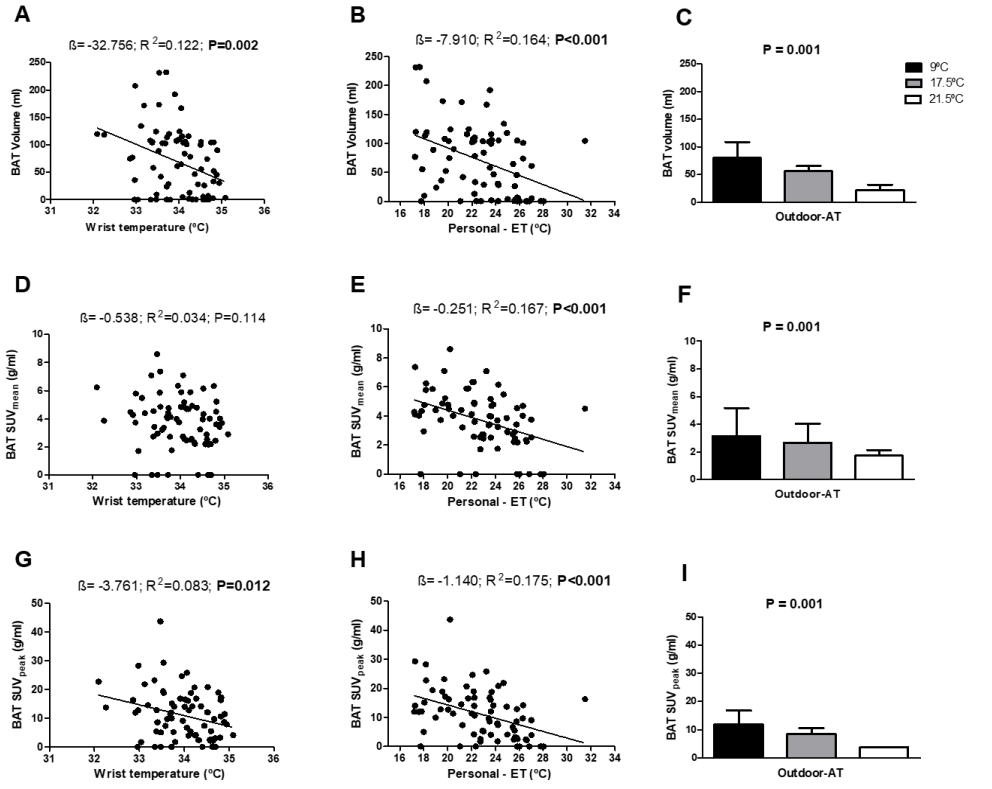

Figure 1. Associations of wrist skin temperature (WT), Personal-environmental temperature (ET), and Outdoor-ambient temperature (AT) with 18F-Fluorodexyglucose (18F-FDG) uptake by brown adipose tissue (BAT) volume and activity. Panels A, B, and C show the associations of WT, Personal-ET, and Outdoor-AT with 18F-FDG uptake by BAT volume, respectively. Panels D, E, and F show the associations of WT, Personal-ET, and Outdoor-AT with 18F-FDG uptake by BAT activity [standardized uptake value (SUVmean)], respectively. Panels G, H, and I show the associations of WT, Personal-ET, and Outdoor-AT with 18F-FDG uptake by BAT activity (SUVpeak), respectively (n=75).

used a one-way analysis of variance (ANOVA) to compare the differences in 18F-FDG uptake by BAT (volume and activity) as well as 18F-FDG uptake by skeletal muscle and descending aorta 18F-FDG uptake across Outdoor-AT categories (9°C, 17.5°C, and 21.5°C). We also conducted bivariate correlations to study the association between 18F-FDG uptake by BAT and skeletal muscle with the number of hours per day in every degree of WT and Personal-ET. We used the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set at <0.05.

RESULTS

Figure 1 shows the associations of WT and Personal-ET with 18F-FDG uptake by BAT volume and activity. We observed modest negative associations of WT and Personal-ET with 18F-FDG uptake by BAT volume (R2=0.122; P=0.002, Figure 1A and R2=0.164; P<0.001, Figure 1B, respectively) and with 18F-FDG uptake by BAT activity (SUVpeak: R2=0.083; P=0.012, Figure 1G and R2=0.175; P<0.001, Figure 1H, respectively). There was also a modest association between Personal-ET and 18F-FDG uptake by BAT activity (SUVmean Figure 1E R2=0.167; P<0.001) but not with WT (R2=0.034; P=0.114). Similarly, the participants exposed to lower Outdoor-AT had higher levels of 18F-FDG uptake by BAT volume and activity [(SUVmean and SUVpeak), all P=0.001, Figure 1C, F, and I].

Figure 2 shows the association of WT and Personal-ET with skeletal muscle 18F-FDG

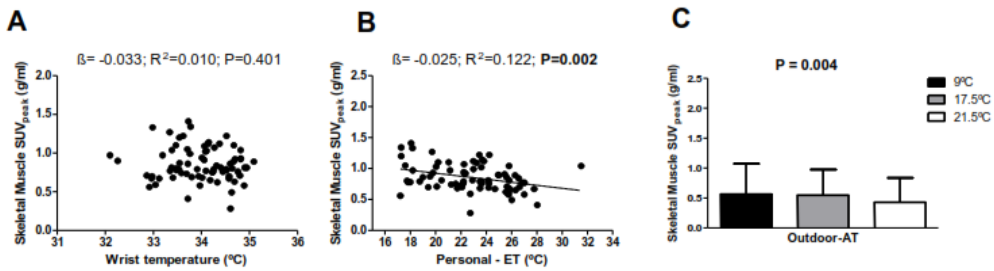

Figure 2. Association of wrist skin temperature (WT) (Panel A), Personal-environmental temperature (ET) (Panel B), and Outdoor-ambient temperature (AT) (Panel C) with 18F-Fluorodexyglucose (*18F-FDG)* uptake by skeletal muscle activity (SUVpeak). Muscle activity is an average of SUVpeak of the paracervical (cervical vertebrae 4), sternocleidomastoid, scalene, longus colli, trapezius, parathoracic (thoracic vertebrae 2), supraspinatus, subscapularis, deltoid, pectoralis major, and triceps braquis muscles, averaged from the right and left sides of the body (n=75).

uptake (SUVpeak). There was no association between WT and skeletal muscle 18F-FDG uptake (R2=0.010; Figure 2A), but a negative and significant association was detected between Personal-ET and skeletal muscle 18F-FDG uptake (R2=0.122; P=0.002, Figure 2B). Similarly, participants exposed to lower Outdoor-AT had higher skeletal muscle 18F-FDG uptake (Figure 2C), and Personal-ET was associated with 18F-FDG uptake by several muscle groupings, especially with deep and cervical muscles (data not shown), but the correlations coefficients are modest. There was no association between WT, Personal-ET, or Outdoor-AT and descending aorta 18F-FDG uptake (all P≥0.144, Figure 3A, B, and C, respectively).

Figure 4 shows descriptive data about the time per day exposed to a certain temperature during 7 days in free day-living conditions in young adults for WT (A) and Personal-ET (B). Figure 5 shows the bivariate correlations of number of hours per day at a certain temperature measured by wrist skin temperature (WT) and Personal-environmental temperature (ET) with 18F-FDG uptake by BAT volume (panel A and E) and activity (panels B, C, F, and G) as well as with 18F-FDG uptake by skeletal muscle activity (panels D and H). The number of hours per day of WT lower than 33°C was positively correlated with 18F-FDG uptake by BAT volume and activity (both SUVmean, SUVpeak, all P≤0.046) whereas the number of hours per day of WT above 35°C was negatively and significantly correlated with 18F-FDG uptake by BAT volume and activity (both SUVmean, SUVpeak, all P≤0.047, Figure 5A, 5B, and 5C, respectively). We observed a positive and significant correlation between the number of hours per day of WT at 33°C and 18F-FDG uptake by skeletal muscle activity (P=0.017, Figure 5D).

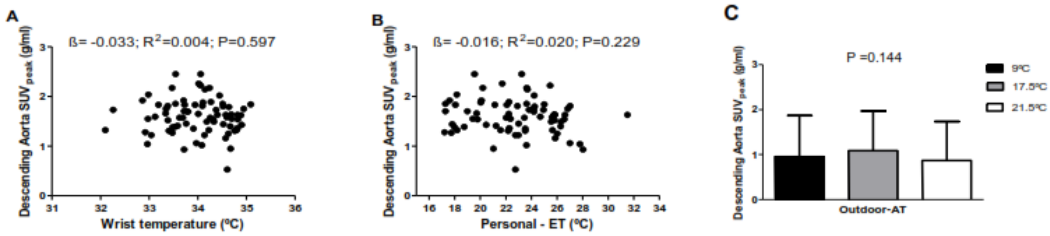

Figure 3. Associations of wrist skin temperature, Personal-environmental temperature (ET), and Outdoor-ambient temperature (AT) with the standardized uptake value (SUV) peak of descending aorta as a reference tissue (n=75).

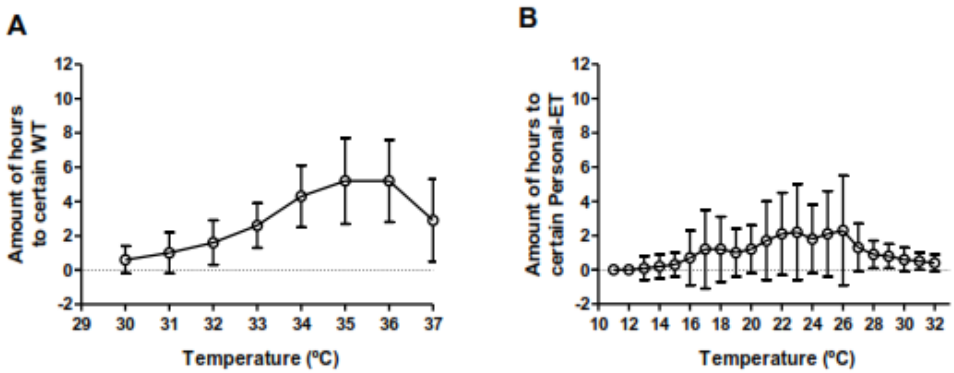

Figure 4. Descriptive data about the amount of hours per day exposed to a certain temperatures during 7 days in free day-living conditions in young adults (n=74). Data are presented as mean and standard deviation (n=75).

The number of hours per day exposed to a Personal-ET between 14°C and 20°C was positively correlated with 18F-FDG uptake by BAT volume and activity (both SUVmean, SUVpeak, Figure 5E, 5F, and 5G, respectively, all P≤0.048). Moreover, we found that the number of hours per day exposed to a Personal-ET between 25°C and 29°C was negatively correlated with 18F-FDG uptake by BAT volume and activity (both SUVmean, SUVpeak, all P≤0.026, Figure 5E, 5F, and 5G, respectively). However, there was no association between the number of hours per day exposed to a Personal-ET between 21°C and 24°C (all P≥0.176). Interestingly, the number of hours per day exposed to Personal-ET between 12°C and 14°C only correlated with skeletal muscle 18F-FDG uptake (all P≤0.039), but not with 18F-FDG uptake by BAT-related outcomes. We also observed that the number of hours exposed to Personal-ET between 26°C and 31°C was negatively correlated with skeletal muscle 18F-FDG uptake (all P≤0.034). Moreover, we did not find any correlation in the ranges lower than 12°C and higher than 32°C with any PET/CT parameters. All the analyses were repeated after adjusting by BMI, LMI, and FMI, and the results persisted (data not shown). We divided the sample by sex and the results did not change (data not shown), even controlling by menstrual cycle and the results persisted (data not shown).

DISCUSSION

The main findings of the present study indicate that WT and Personal-ET were negatively associated with BAT volume and activity and with skeletal muscle activity as measured by the 18F-FDG-PET/CT scan, although these associations are relatively weak and the results should be interpreted with caution. In addition, the time per day that participants were exposed to a temperature between 14-20°C was positively correlated with BAT volume and activity; however, when the range of temperature was between 12 and 14°C, the correlation with 18F-FDG uptake by BAT disappeared and became significant with 18F-FDG uptake by skeletal muscle activity. Furthermore, we also found a range of warm temperature (≥24°C) which could inhibit 18F-FDG uptake by BAT, as well as skeletal muscle thermogenesis. Here, we show that WT and Personal-ET are informative markers of the real exposure of the participants previous BAT quantification; however, based on the weak correlation coefficients further studies are needed.

WT and Personal-ET to control the seasonal variation of 18F-FDG uptake

To date, studies investigating the seasonal variation of human BAT metabolism have

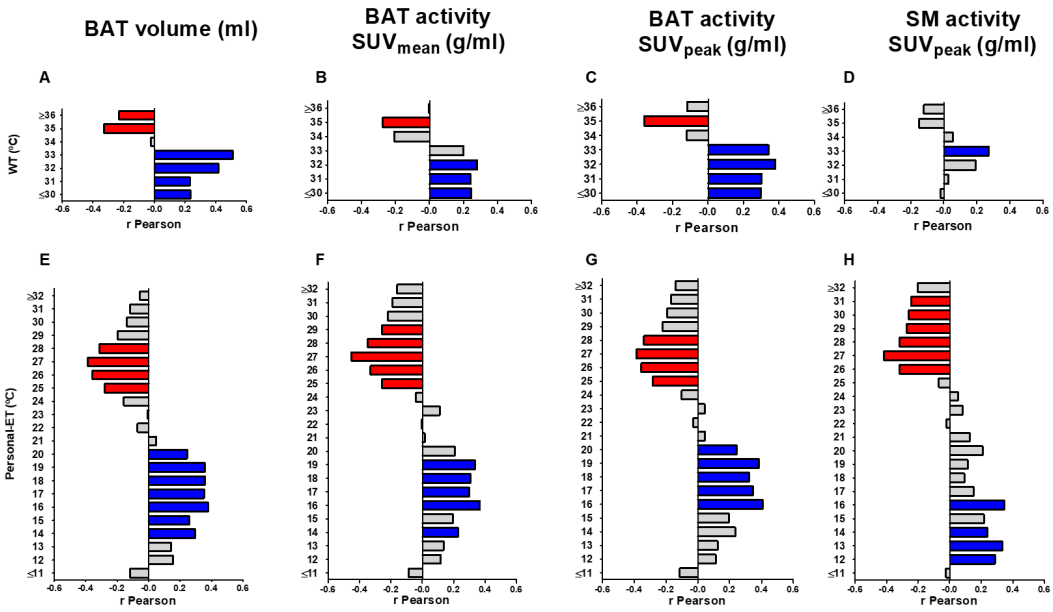

Figure 5. Bivariate correlations of number of hours per day at a certain temperature measured by wrist skin temperature (WT) and Personal-environmental temperature (ET) with 18F-Fluorodexyglucose (*18F-FDG)* uptake by BAT volume (panel A and E) and activity (panels B, C, F, and G) as well as with skeletal muscle activity (panels D and H). Each Celsius degree represents temperatures in the whole range for instance, 13°C Personal-ET contains the ranges of temperature from 13.00 to 13.99°C. Blue bars represent positive and significant (P<0.05) correlations whereas red bars represent negative and significant (P<0.05) correlations (n=75).

used either the date when the PET/CT scan was performed [12,17] or the temperature recorded by a local meteorological agency [9,10,30]. However, Outdoor-AT data could not be representative of the individual's daily exposure. It is likely that when temperatures are low outdoor, people spend more time indoor at more comfortable temperatures [31]. Thus, WT and Personal-ET are more representative values of the real daily ambient temperature to which people are exposed. Based on the associations between WT and Personal-ET and 18F-FDG uptake by BAT and skeletal muscle activity, it seems that researchers should probably use these measurements as a cofounding variable when studying the effect of an intervention (e.g. pharmacological) on 18F-FDG uptake by BAT volume and activity and/or skeletal muscle activity. Based on our data, we do not recommend the use of the WT, because the explained variance is lower in comparison to Personal-ET, and because WT could be influenced by the individual's thermoregulatory capacity [20,32]. Nevertheless, researchers should select which measurement (WT or Personal-ET) is more convenient to answer the scientific question.

18F-FDG uptake by BAT and skeletal muscle in colder temperatures

18F-FDG is taken up by members of the sodium-independent glucose transport family (GLUT) and is then phosphorylated by hexokinase to the 6-phospate, which is trapped intracellularly [33]. BAT and skeletal muscles share GLUT4 to trap the 18F-FDG intracellularly. Recently, Hanssen et al. [16] observed that 10 days of cold-air acclimation at 14-15°C led to an enrichment of GLUT4 at the sarcolemma of the skeletal myocytes, and suggested that this mechanism could explain why they found that the 18F-FDG uptake in the skeletal muscles increased after the cold intervention. Therefore, it is biologically plausible that different ranges of

cold or warm-temperatures modify GLUT4 translocation in BAT or skeletal muscle in different ways. Lee et al. [15] showed that BAT presents a plasticity (based on the 18F-FDG uptake) at different air cold-acclimation (19°C vs. 24°C), showing that BAT could adapt its 18F-FDG uptake after different air cold-acclimation, whereas the rectus femoralis did not show any alteration in the 18F-FDG uptake. These findings could be based on: (i) the cold stimuli was not strong enough to increase the skeletal muscle 18F-FDG uptake or to involve this tissue in the thermogenic response, as we postulate in the present study (Figure 5), (ii) the relatively small sample size of the study (i.e. 5 participants) and/or, (iii) the anatomical location of the rectus femoralis in the lower part of the body, which does not seem to be directly involved in shivering [29]. Blondin et al. [29] showed that deep skeletal muscles of the neck or psoas iliac have a higher 18F-FDG uptake after a cold-induced PET/CT scan. These findings showed that the 18F-FDG uptake by these skeletal muscles, which have physiological and functional roles, seems to be firstly involved in shivering in comparison to others.

We have observed that BAT and skeletal muscle 18F-FDG uptake are associated with different ranges of cold temperatures. These observational findings could support the idea that the thermogenic responses occur in the human body hierarchically, being BAT the first agent involved in maintaining the core body temperature constant during the first phases of cold exposure. It seems that in colder exposures skeletal muscles must also be involved [32]. Based on the results of our study, we postulate that BAT is activated when humans are exposed to temperatures below thermoneutrality (<20°C) [34]. If the temperature continues decreasing (around 15°C), skeletal muscles seem to be activated and contribute to the thermogenesis, possibly by the sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA) [5]. If the ambient temperature continues decreasing, then skeletal muscles start shivering as a last resource to generate heat to keep the core temperature constant [32].

In agreement with our findings, it is likely that at temperatures below 13-14 °C, BAT does not contribute as much to defend the body temperature. In the study of U Din et al. [8], the cooling protocol performed before the PET scans was conducted with a very cold temperature (cooling blankets at 6°C during 2 hours) and with a dynamic PET scan and therefore, this could be the reason because they observed that deep skeletal muscles were the major contributor to CIT. Further studies are needed with higher temperatures and using dynamic PET scans in order to understand the ranges of cold temperatures activating or inducing BAT and skeletal muscle glucose uptake, as well as to discern the main tissue involve in CIT. In a separate study [35], we showed a significant increase of fat oxidation during the first 30 minutes of cold expose, which was gradually decreased thereafter. Whether this fact is linked to BAT activation as a first agent to prevent core body temperature is however not known [35]. In animal models, Rowland et al. [34] showed that knocking out the gene encoding sarcolipin, which eliminates a potential contribution of skeletal muscles to thermogenesis, results in a compensatory increase in UCP1 in BAT and beige adipose tissue. In contrast, knockout of UCP1 is compensated by increased levels of sarcolipin in muscle, which indicates synergy of both thermogenic mechanisms in vivo [36]. Our findings suggest that both BAT and skeletal muscles contribute additively to thermogenesis in humans, yet, further studies are warranted.

18F-FDG uptake by BAT and skeletal muscle in warmer temperatures

Lee et al. [15] showed that human BAT has the possibility to be inhibited (at least decrease its 18F-FDG uptake) in an ambient temperature of 24°C. Several studies showed that in summer or hot/warm environments the BAT 18F-FDG uptake decreased in comparison to cold ambient [17,37]. Therefore, it remains of interest to elucidate at which temperature (or range of) human BAT thermogenesis is inhibited in free day

living conditions [17,37]. Here, we showed that when humans spent the most of their daytime above 24°C, both BAT and skeletal muscles glucose uptake is diminished, which concur with the study by Lee et al. [15]

Limitations

The results of this study should be considered with caution, since this is an observational study and it was performed in the south of Spain during 2 months where, the outdoor temperature variation was not high enough (9-21.5°C). Moreover, the correlations found were relatively low. For logistical reason, we were not able to control the clothing of the participants during the measurement of Personal-ET and WT. We performed a personalized cooling protocol, which have several limitations [38]. Moreover, we know that during sleep phases humans can lose at least 25% of their total thermoregulatory capacity. Moreover, we know that during sleep phases humans can lose at least 25% of their total thermoregulatory capacity. However, excluding the sleep phase did not alter the results (data not shown). We do not know whether these findings apply to older participants. In addition, we determined the uptake of 18F-FDG by BAT and skeletal muscles. This may not necessarily reflect substrate utilization by these tissues, as brown adipocytes preferentially consume fatty acids [39], or the metabolic activity of these tissues, which ideally is quantified with tracers such as 15O-O2 or 11C-acetate [8].

CONCLUSIONS

The present study shows that both WT and Personal-ET were negatively associated with BAT and skeletal muscle 18F-FDG uptake. Moreover, we show that the number of hours per day exposed to different ranges of cold temperatures were differently correlated with BAT and skeletal muscles 18F-FDG uptake, showing that during daily living conditions 18F-FDG uptake by BAT and skeletal muscles seem to be involved in the thermoregulatory system hierarchically, although the correlation coefficients are weak. Moreover, we observed a range of warm temperature (≥24°C) that could inhibit both 18F-FDG uptake by BAT and skeletal muscle. However, these results suggest that Personal-ET can be used as a co-variable, as well as WT, to estimate the personalized ambient temperature exposure for each participant before human BAT and skeletal muscle quantification with 18F-FDG-PET/CT

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The mediating role of brown adipose tissue and skeletal muscle measured by 18F-Fluorodeoxyglucose in the thermoregulatory system in young adults

CHAPTER 10

BACKGROUND

The regulation of core body temperature is one of the most critical functions of the human body [1]. Core body temperature is regulated by behavioral and physiological mechanisms [1,2]. Behavioral strategies are voluntary and oriented responses that help to maintain core body temperature, such as modifying posture, wearing clothing in winter, or using cold-air-conditioning in summer [2]. On the other hand, physiological mechanisms are involuntary responses that generate or dissipate heat. In mammals, four physiological mechanisms are particularly involved in thermoregulation [1]: (i) water evaporation (sweating), (ii) control of the skin blood flow, (iii) non-shivering thermogenesis (NST), and (iv) shivering thermogenesis. These mechanisms constantly interact, and their main aim is to keep the core body temperature in a normal range.

Skin temperature is a feedforward mechanism of the thermoregulatory system [1]. When a change in the ambient temperature is detected by skin thermoreceptors, these trigger thermoregulatory responses that prevent any change in core body temperature [3]. When humans are exposed to warm environments, peripheral blood vessels are dilated in order to promote heat loss (vasodilation), whereas in cold environments, peripheral blood vessels are constricted to prevent heat loss (vasoconstriction) [1]. In animals, the engagement of specific thermoregulatory strategies is hierarchical [4]. For instance, vasoconstriction occurs before NST, because vasoconstriction energy efficiency is higher than NST activation at least in mice models [4,5]. However, whether skin blood flow regulation mechanisms work hierarchically or concomitantly with NST activation or inhibition has not yet been studied in humans.

Both brown adipose tissue (BAT) and some skeletal muscles [6] play a role in NST. BAT is a specialized tissue for the rapid production of heat when the body is exposed to cold temperatures, which is mediated by the action of the uncoupling protein 1 [7]. In humans, BAT is mainly metabolically active upon cold exposure [8–10]. However, BAT consume large quantities of energy expenditure in small mammals, although its contribution to NST in humans seems to be negligible, being the skeletal muscle the main effector of NST [6,11,12] and shivering (muscle contractions) during cold exposure [13]. However, the contribution of BAT and skeletal muscle in the regulation of thermogenesis is largely unknown [6,14,15].

There are several ways to assess environmental temperature exposure [16,17]. Some studies quantified the personal level of environmental temperature (Personal-ET) [17], measured by an iButton during a period of 7 days. This iButton is always with the participant and should be in direct contact with the air (never with the skin). This is thus a surrogate marker of temperature exposure of every individual. Other studies [18,19] quantified a proxy of skin blood flow mechanisms [20,21] and chronobiology [22] outcomes attaching an iButton to the wrist, measuring the wrist temperature (WT), normally at the same time that the Personal-ET. Personal-ET is related to WT [17]; however, since cold and warm exposures have a direct effect on activation or inhibition of BAT and skeletal muscle, it could be that these thermogenic tissues might have a mediating role between Personal-ET and WT.

Based on the aforementioned, we studied the mediating role of BAT and skeletal muscle activity [assessed by cold-induced 18F-Fluorodeoxyglucose (18F-FDG) uptake] between Personal-ET and WT in young healthy adults for 7 days (24 hours/day). In order to understand the physiological mechanisms, we examined whether the association of the number of hours exposed to a certain Personal-ET with BAT and skeletal muscle 18F-FDG uptake is mediated by WT as a surrogate marker of skin blood flow mechanisms.

MATERIAL AND METHODS

A total of 90 (n=65 women) white Caucasian healthy adults aged 21.9 ± 2.3 years old participated in the present study (Table 1). The participants were enrolled in the ACTIBATE study [23], an exercise-based randomized controlled trial (Clinical Trials.gov

ID: NCT02365129). All participants were non-smokers, were not enrolled in a weight loss program, had a stable body weight (body weight changes <3 kg) over the previous 3 months, were not physically active (<20 minutes on <3 days/week), did not take any medication, had no acute or chronic illness, and reported not to be regularly exposed to cold. The study was conducted in Granada (Southern Spain) between October and November in 2015 and 2016. The study protocol and informed consent were conducted in accordance with the Declaration of Helsinki (revision of 2013), and they were approved by the Human Research Ethics Committee of both the University of Granada (n° 924) and the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada). A written informed consent was obtained from all the participants.

Wrist and Personal Environmental temperatures measurements

All participants wore 2 iButtons (DS-1922 L, Thermochron; resolution: 0.0625 °C; frequency: 10 min intervals; Maxim, Dallas, USA) for 7 days. One iButton was placed on the ventral side of the wrist of the non-dominant hand over the radial artery with a wrist band in order to determine WT. We instructed the participants to wear the iButton on the wrist for the whole day (even when asleep) and to take it off only when bathing or swimming. A second iButton was attached to a plastic fob and was used to quantify the Personal-ET. This iButton remained with the participant at all times but was never in direct contact with the body [17] or under clothing. During sleep-phases, the Personal-ET sensor was placed on the bedside table. The iButtons were programmed to start the recording at 06.00 and to finish 7 days later at 12.00 in the morning when the 18F-FDG positron emission tomography in combination with a computed tomography scan (18F-FDG) positron emission tomography with computed tomography (PET/CT) scan was performed. The participants registered the non-wear periods in a diary during the 7 days. We excluded the non-wear periods as well as those participants with less than 5 valid days. For a day to be considered valid at least 75% of the day had to be registered (≥18 hours). All iButtons were programmed and analyzed with the Temperatus® software (http://profith.ugr.es/temperatus?lang=en). We calculated an average of the valid recordings for the 7 days for both WT and Personal-ET separately. Moreover, we calculated the number of hours per day that the participants were exposed to a certain temperature with a 1°C-range from 11 to 42°C for the Personal-ET (e.g. 11-11.99°C, 12-12.99°C, etc.) and from 29 to 37°C for WT (e.g. 29-29.99°C, 30-30.99°C, etc.).

Personalized cooling protocol

The personalized cooling protocol has been explained in detail elsewhere [24]. Briefly, the participants entered a mild-cold room (around 19.5°C), and they were asked to wear a water perfused cooling vest (Polar Products Inc., Ohio, USA). We determined the participant's shivering threshold, reducing the water temperature gradually until shivering occurred. Shivering was determined both visually by researchers as well as self-reported by the participants. After 48-72 hours, we exposed the participants to 2 hours at their personalized temperature to induce maximum non shivering thermogenesis (above ~4°C) [25]. After 1 hour of cold exposure, we injected a bolus of 18F-FDG (~185MBq), and we increased the water temperature 1°C in order to prevent shivering. After 2 hours of cold exposure we performed the PET/CT scan from the atlas vertebrae to the thoracic vertebra 6. The evaluations were performed in 4 different weeks among 2 months (from October to November 2016) in Granada, Spain.

Quantification of 18F-FDG uptake by BAT and skeletal muscle

We quantified BAT volume and activity following the recently published recommendations [26]. PET/CT images were analyzed using the Beth Israel plugin for FIJI [24] software. We applied an individualized standardized uptake value (SUV) threshold

[1.2/(lean body mass/body mass)] [26] with a fixed range of Hounsfield units (HU, −190 to −10). We quantified BAT volume and activity (i.e. SUVmean, SUVpeak). We computed BAT metabolic activity as BAT volume*SUVmean [24] as well as the 18F-FDG uptake by a reference tissue (descending aorta). We quantified the 18F-FDG uptake (SUVpeak) of several skeletal muscles between the atlas vertebrae and the thoracic vertebra 4. We drew a single region of interest (ROI) from 1 slice in paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii muscles from both left and right sides of the body [11,27]. An average of both sides including all skeletal muscles was calculated in order to obtain a single representative value of the skeletal muscle glucose uptake of the upper part of the body. Our protocol has shown a high inter-observer reliability, regardless of the threshold applied to quantify BAT [28].

Body composition

Body composition was assessed on a separate day by Dual Energy X-ray Absorptiometry (HOLOGIC, Discovery Wi) [23]. The participants' weight and height were measured without shoes and wearing a T-shirt and shorts using a SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany), and we calculated body mass index (BMI) (kg/m2).

Statistical analysis

The descriptive characteristics of the study sample are presented as mean and standard deviation (SD) unless otherwise stated. There was no sex interaction (all P>0.10) in any of the study variables, thus we conducted the analyses in men and women together.

To quantify the mediating role of BAT volume, activity (i.e. SUVmean, SUVpeak), and metabolic activity, and skeletal muscle activity in the relationship between Personal-ET and WT, we conducted mediation analyses [29]. In addition, we tested the mediating role of WT on the association of the number of hours per day exposed to a certain Personal-ET with BAT volume and activity, and skeletal muscle activity. We used the PROCESS macro version 3.0, model four, with 5.000 bias-corrected bootstrap samples and 95% confidence intervals. Bootstrapping is a nonparametric resampling procedure which does not require the assumption of normality of the sampling distribution [30]. The mediation was estimated using the indirect effect, which indicates the change on the effect of the independent variable on the outcome that can be endorsed to the proposed mediator. Indirect effects (a*b paths) with confidence intervals not including zero are interpreted as statistically significant [31], which could occur regardless of the significance of the total effect (c path, effect of the independent variable on the dependent variable) and the direct effect (c' path, effect on the dependent variable when both the independent and the mediator variables are included as independent variables) [29]. To quantify how much of the total effect was due to the mediation, we calculated the percentage of mediation [(indirect effect / total effect) x 100], provided when the total effect was larger than the indirect effect with the same direction [29]. All the analyses were performed using the IBM SPSS Statistics for Windows version 22.0 (Armonk, NY: IBM Corp), and the level of significance was set at P<0.05.

RESULTS

Table 1 shows the characteristics of the participants. A total of 15 out of 90 participants were excluded because less than 5 valid days of temperature had been recorded. A total of 75 participants (74.6% women) were finally included in the analyses, with 6.3 ± 0.5 valid days. The average age was 21.9 ± 2.3 years old and with a BMI of 25.2 ± 4.8 kg/m^2.

Table 1. Characteristics of the study participants

	n=75
Sex (% women)	74.6%
Age (years)	21.9 ± 2.3
Body mass index (kg/m2)	25.2 ± 4.8
Lean mass (kg)	41.3 ± 9.6
Fat mass (kg)	26.9 ± 9.5
Fat mass (%)	37.6 ± 7.0
BAT volume (ml)	69.0 ± 61.3
BAT activity (SUVmean g/ml)	3.7 ± 1.9
BAT activity (SUVpeak g/ml)	11.0 ± 8.5
Wrist Temperature (°C)	34.0 ± 0.7
Personal-ET (°C)	22.8 ± 3.1

Data are presented as mean and standard deviation, unless otherwise stated. BAT: brown adipose tissue, Personal-ET: Personal level of environmental temperature, SUV: standardized uptake value.

The mediating role of BAT

Figure 1 shows the mediating effect of BAT volume, activity (i.e. SUVmean and SUVpeak), BAT metabolic activity, and skeletal muscle activity (SUVpeak) in the relationship between Personal-ET and WT. Personal-ET was positively associated with WT (c path= 0.0763; P=0.0014) and negatively associated with BAT-related outcomes (volume, SUVmean, SUVpeak, and metabolic activity, a path, all P<0.001, see Figure 1 panels A, B, C, D, respectively) and skeletal muscle activity (a path, P=0.0023, see Figure 1E). BAT-related outcomes and skeletal muscle activity were not significantly associated with WT (b path). After including the mediator variables in the model (see Figure 1 c´ path; all P<0.05), the direct effect of Personal-ET on WT remained statistically significant. The percentages of mediation of BAT volume and metabolic activity in the relationship between Personal-ET and WT were 25.4% and 23.9%, respectively. However, we did not observe any mediating effect of BAT activity (i.e., SUVmean and SUVpeak) and skeletal muscle activity in the relationship between Personal-ET and WT (see Figure 1 panels: B, C, and E, respectively). These results persisted after controlling for sex, BMI, FMI, or LMI (data not shown). Furthermore, we repeated the analyses using BAT-related outcomes as well as skeletal muscle activity multiplied by lean body mass percentage [32] and the results remained unchanged (data not shown).

The mediating role of WT

Figure 2A shows the mediating effect of WT in the relationship between the number of hours exposed to a certain Personal-ET and BAT-related outcomes (volume, SUVpeak, and metabolic activity). The number of hours per day exposed to a warm Personal-ET was negatively associated with BAT volume (from 25°C to 28°C; c path; all P<0.05) and positively associated with WT (from 24°C to 27°C; a path; all P<0.05) (Table 2). WT was also negatively associated with BAT volume at this temperature range (b path; all P<0.05). The direct effect was only significant when examining the number of hours per day exposed to temperatures ≥26°C (c' path; all P<0.05) (Table 2). WT showed the highest percentage of mediation (57%) in the relationship between the number of hours exposed to 24°C and BAT volume in comparison with other ranges of warm temperatures (see Figure 2E). In addition, we observed that the number of hours per day exposed to a cold Personal-ET was positively related to BAT volume (from 14°C to 20°C; c path; all P<0.05) and negatively associated with WT (from 16°C to 20°C; a path; all P<0.05) (Table 3). WT was negatively associated with BAT volume (b path; P<0.05) and the association between the number of hours exposed to a cold temperature (from 16°C to 19°C) and BAT volume persisted after including WT as a mediator (c' path; both; all P<0.05). The sign of the indirect effect changed during the ambient exposure, being positive during cold-ambient exposure and negative during warm-ambient exposures (see Figure 2B-G). Moreover, when the participants were exposed to a certain range of temperature in the thermoneutral zone,

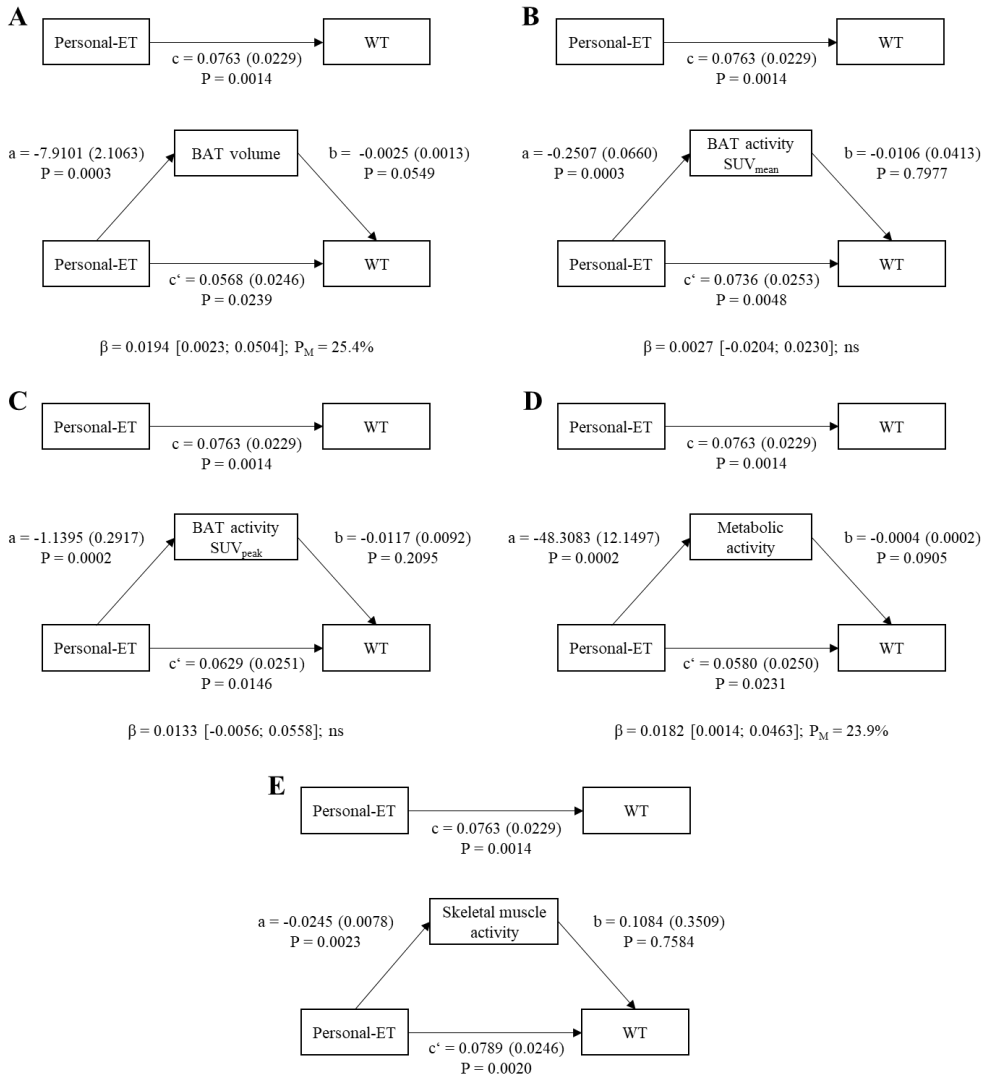

Figure 1. Mediation models of the relationship between personal levels of environmental temperature and wrist skin temperature with (A) BAT volume (ml), (B) SUVmean (g/ml), (C) SUVpeak (g/ml), (D) metabolic activity (calculated as BAT volume x BAT SUVmean), and (E) skeletal muscle activity (g/ml) included as mediator variables, respectively. Paths a, b, c, and c' are presented as unstandardized coefficients (Standard Error, SE). β = indirect effect (a*b paths); [lower limit confident interval: upper limit confident interval], lower and upper levels for 95% confidence interval of the indirect effect based on 5000 bootstraps.

WT did not play a mediating role in BAT volume (from 21°C to 23°C, see Figures 2B and E). The mediation analyses were performed for the number of hours exposed to each degree of Personal-ET showing that the mediating effect disappeared at temperatures ≥28°C or ≤14°C, probably due to a lack of statistical power at these ranges (small number of participants exposed to these extreme temperatures). The mediating role of WT was also observed in the relationship between Personal-ET and BAT activity (i.e. SUVpeak and metabolic activity, see Figure 2 for the indirect effect: panels C and D, respectively, and for the percentage of mediation: panels F and G, respectively; see

Table 2. Total, direct, and indirect effects of the simple mediation analyses investigating the mediating role of wrist temperature in the association between the number of hours exposed to a certain personal environmental temperature and brown adipose tissue volume.

Independent variable	Total effect (*c*)	Direct effect (*c'*)	Path *a*	Path *b*	Indirect effect (*a*b*)	BC 95% CI Lower Upper	P_M (%)
Number of hours per day exposed to							
28°C	-24.1511 (8.5478)**	-20.7341 (8.2251)*	0.1172 (0.0952)	-29.1556 (10.0126)**	-3.4170	-13.6843; 2.2662	
27°C	-17.5167 (4.8821)***	-14.2999 (4.9035)**	0.1296 (0.0545)*	-24.8155 (10.1490)*	**-3.2168**	-8.9958; -0.5952	18.36
26°C	-6.5085 (1.9827)**	-4.8443 (2.0725)*	0.0702 (0.0211)**	-23.7003 (10.6935)*	**-1.6642**	-3.4272; -0.3454	25.57
25°C	-7.2114 (2.8879)*	-4.9839 (2.9130)	0.0814 (0.0307)**	-27.3753 (10.6111)*	**-2.2275**	-5.4156; -0.4822	30.89
24°C	-5.0423 (3.6912)	-2.1706 (3.6547)	0.0925 (0.0384)*	-31.0297 (10.7150)**	**-2.8717**	-5.8755; -0.9852	56.95
23°C	-0.1435 (2.5060)	0.9153 (2.3847)	0.0318 (0.0265)	-33.3129 (10.4289)**	-1.0588	-2.6913; 0.0416	
22°C	-1.7610 (2.8817)	-1.7698 (2.7173)	-0.0003 (0.0309)	-32.7633 (10.3079)**	0.0087	-1.6455; 2.1567	
21°C	1.5069 (3.5844)	-0.0470 (3.4213)	-0.0474 (0.0379)	-32.7773 (10.4483)**	1.5539	-0.6785; 4.8597	
20°C	10.8615 (5.0206)*	6.9503 (5.0396)	-0.1373 (0.0529)*	-28.4826 (10.6645)**	**3.9112**	1.1979; 9.3971	36.01
19°C	17.0622 (5.2043)**	13.6895 (5.1955)*	-0.1305 (0.0575)*	-25.8529 (10.2149)*	**3.3727**	0.5235; 9.4524	19.77
18°C	10.7468 (3.2756)**	7.9320 (3.4450)*	-0.1199 (0.0347)***	-23.4791 (10.7603)*	**2.8148**	0.5623; 8.0286	26.19
17°C	9.0101 (2.7922)**	6.1971 (3.0982)*	-0.1271 (0.0282)***	-22.1406 (11.3763)	**2.8130**	0.3676; 6.3115	31.22
16°C	14.1431 (4.0529)***	11.5538 (4.0487)**	-0.1021 (0.0452)*	-25.3649 (10.1354)*	**2.5893**	0.4701; 6.8149	18.31
15°C	21.6970 (9.6794)*	16.0500 (9.5090)	-0.2054 (0.1042)	-28.8089 (10.4058)**	**5.9170**	1.0768; 16.2034	26.94
14°C	23.2957 (8.7497)**	23.1941 (8.2016)**	-0.0031 (0.0979)	-32.6534 (9.8079)**	0.1015	-8.4056; 8.6450	

Results shown as unstandardized coefficients (Standard Error, SE), and bias corrected (BC) 95% confidence interval (CI) of the indirect effect based on 5000 bootstraps.
BC: Bias corrected; CI: confidence interval; P_M: percentage of mediation.
Statistical significant indirect effects indicating that 0 is not in the 95% confidence interval (CI) of the indirect effect are presented in bold.
p-values indicating associations between study variables = *P<0.05, **P<0.01, ***P<0.001.

Table 3 and 4 for further details). Furthermore, we did not find a mediating effect of the WT on the association of the number of hours exposed to a certain Personal-ET with SUVmean and skeletal muscle activity (data not shown), as well as in upper (>29°C) and lower (<13°C) ranges of temperature due to the lack of statistical power in these ranges (data not shown). The results persisted after controlling by sex, BMI, LMI, FMI, or date when the evaluation were performed (data not shown). Overall, the results persisted, when we repeated all the analyses excluding data regarding the temperature ranges, for both WT and Personal-ET, when the participants were asleep (data not shown). Moreover, we repeated the analyses using other classifications of skeletal muscles [11] activity (SUVpeak) and the absence of a mediating role of this tissue persisted (data not shown).

Table 3. Total, direct, and indirect effects of the simple mediation analyses investigating the mediating role of wrist temperature in the association between the number of hours exposed to a certain personal environmental temperature and standardized uptake value peak.

Independent variable	Total effect (*c*)	Direct effect (*c'*)	Path *a*	Path *b*	Indirect effect (*a*b*)	BC 95% CI Lower Upper	P_M (%)
Number of hours per day exposed to							
28ºC	-3.6344 (1.1794)**	-3.2599 (1.1593)**	0.1172 (0.0952)	-3.1947 (1.4113)*	-0.3744	-1.7238; 0.2237	
27ºC	-2.4402 (0.6799)***	-2.1041 (0.6951)**	0.1296 (0.0545)*	-2.5924 (1.4387)	**-0.3360**	-1.0857; -0.0057	13.77
26ºC	-0.8976 (0.2765)**	-0.7293 (0.2937)*	0.0702 (0.0211)**	-2.3975 (1.5152)	-0.1683	-0.4264; 0.0264	
25ºC	-1.0210 (0.4016)*	-0.7839 (0.4128)	0.0814 (0.0307)**	-2.9145 (1.5036)	**-0.2372**	-0.6959; -0.0130	23.23
24ºC	-0.4558 (0.5178)	-0.1163 (0.5213)	0.0925 (0.0384)*	-3.6683 (1.5283)*	**-0.3395**	-0.7859; -0.0673	74.49
23ºC	0.1326 (0.3487)	0.2571 (0.3384)	0.0318 (0.0265)	-3.9171 (1.4800)**	**-0.1245**	-0.3651; -0.0015	N/A
22ºC	-0.1011 (0.4022)	-0.1021 (0.3877)	-0.0003 (0.0309)	-3.7612 (1.4708)*	0.001	-0.2129; 0.2276	
21ºC	0.1960 (0.4993)	0.0180 (0.4870)	-0.0474 (0.0379)	-3.7529 (1.4872)*	0.1779	-0.0555; 0.6272	
20ºC	1.5091 (0.6993)*	1.0842 (0.7154)	-0.1373 (0.0529)*	-3.0942 (1.5139)*	**0.4249**	0.0396; 1.1213	28.16
19ºC	2.5401 (0.7171)***	2.1937 (0.7299)**	-0.1305 (0.0575)*	-2.6546 (1.4351)	**0.3463**	0.0104; 1.1236	13.63
18ºC	1.3436 (0.4626)**	1.0383 (0.4931)*	-0.1199 (0.0347)***	-2.5464 (1.5403)	**0.3053**	0.0136; 0.9144	22.72
17ºC	1.2252 (0.3901)**	0.9553 (0.4389)*	-0.1271 (0.0282)***	-2.1244 (1.6115)	0.2699	-0.0720; 0.7686	
16ºC	2.1308 (0.5563)***	1.8689 (0.5667)**	-0.1021 (0.0452)*	-2.5652 (1.4187)	**0.2619**	0.0080; 0.7930	12.29
15ºC	2.3449 (1.3674)	1.6561 (1.3661)	-0.2054 (0.1042)	-3.3535 (1.4950)*	**0.6888**	0.0485; 2.0564	29.37
14ºC	2.5672 (1.2404)*	2.5555 (1.1931)*	0.1172 (0.0979)	-3.7495 (1.4268)*	0.0117	-0.9257; 0.9111	

Results shown as unstandardized coefficients (Standard Error, SE), and bias corrected (BC) 95% confidence interval (CI) of the indirect effect based on 5000 bootstraps.
BC: Bias corrected; CI: confidence interval; P_M: percentage of mediation; N/A: non-applicable according to statistical assumptions specified previously.
Statistical significant indirect effects indicating that 0 is not in the 95% confidence interval (CI) of the indirect effect are presented in bold.
p-values indicating associations between study variables = *P<0.05, **P<0.01, ***P<0.001.

Table 4. Total, direct, and indirect effects of the simple mediation analyses investigating the mediating role of wrist temperature in the association between the number of hours exposed to a certain personal environmental temperature and metabolic activity.

Independent variable	Total effect (*c*)	Direct effect (*c'*)	Path *a*	Path *b*	Indirect effect (*a*b*)	BC 95% CI Lower Upper	P_M (%)
Number of hours per day exposed to							
28ºC	-142.2746 (49.6038)**	-123.6264 (48.0546)*	0.1172 (0.0952)	-159.1181 (58.4978)**	-18.6482	-76.7198; 13.6812	
27ºC	-92.7638 (28.7954)**	-74.7342 (29.0376)*	0.1296 (0.0545)*	-139.0875 (60.1009)*	**-18.0296**	-50.4684; -2.6810	19.44
26ºC	-34.8199 (11.6524)**	-25.4847 (12.2166)*	0.0702 (0.0211)**	-132.9461 (63.0354)*	**-9.3352**	-19.6154; -1.8476	26.81
25ºC	-41.1781 (16.8086)*	-29.0344 (17.0508)	0.0814 (0.0307)**	-149.2399 (62.1105)*	**-12.1436**	-31.4411; -2.6588	29.49
24ºC	-31.1133 (21.4178)	-15.5447 (21.3620)	0.0925 (0.0384)*	-168.2222 (62.6304)**	**-15.5686**	-32.7741; -4.8195	50.04
23ºC	-2.0202 (14.5630)	3.7930 (13.9632)	0.0318 (0.0265)	-182.8941 (61.0639)**	**-5.8132**	-15.5371; -0.0279	N/A
22ºC	-8.0621 (16.7643)	-8.1103 (15.9204)	-0.0003 (0.0309)	-180.6196 (60.3934)**	0.0482	-9.8282; 11.9435	
21ºC	6.3637 (20.8440)	-2.2449 (20.0209)	-0.0474 (0.0379)	-181.5810 (61.1410)**	8.6086	-3.0637; 29.3434	
20ºC	54.4724 (29.4166)	32.4111 (29.6347)	-0.1373 (0.0529)*	-160.658 (62.7118)*	**22.0613**	6.3974; 55.8110	40.5
19ºC	93.9165 (30.4749)**	75.3119 (30.5755)*	-0.1305 (0.0575)*	-142.6087 (60.1146)*	**18.6046**	2.8291; 52.5241	19.81
18ºC	56.6625 (19.2841)**	40.7224 (20.3314)*	-0.1199 (0.0347)***	-132.9587 (63.5043)*	**15.9400**	3.4256; 45.2335	28.13
17ºC	50.0828 (16.3261)**	34.6886 (18.1741)	-0.1271 (0.0282)***	-121.1656 (66.7343)	**15.3941**	1.9813; 38.6296	30.74
16ºC	98.7912 (22.6644)***	85.9709 (22.8526)***	-0.1021 (0.0452)*	-125.5885 (57.2095)*	**12.8203**	2.8726; 34.6266	12.98
15ºC	127.3577 (56.2649)*	95.0696 (55.6223)	-0.2054 (0.1042)	-157.2056 (60.8681)*	**32.288**	5.3280; 88.6667	25.35
14ºC	162.8793 (49.7349)**	162.3202 (46.8374)***	-0.0031 (0.0979)	-179.8669 (56.0109)**	0.5591	-40.2083; 48.2821	

Results shown as unstandardized coefficients (Standard Error, SE), and bias corrected (BC) 95% confidence interval (CI) of the indirect effect based on 5000 bootstraps.
BC: Bias corrected; CI: confidence interval; P_M: percentage of mediation; N/A: non-applicable according to statistical assumptions specified previously.
Statistical significant indirect effects indicating that 0 is not in the 95% confidence interval (CI) of the indirect effect are presented in bold.
p-values indicating associations between study variables = *P<0.05, **P<0.01, ***P<0.001.

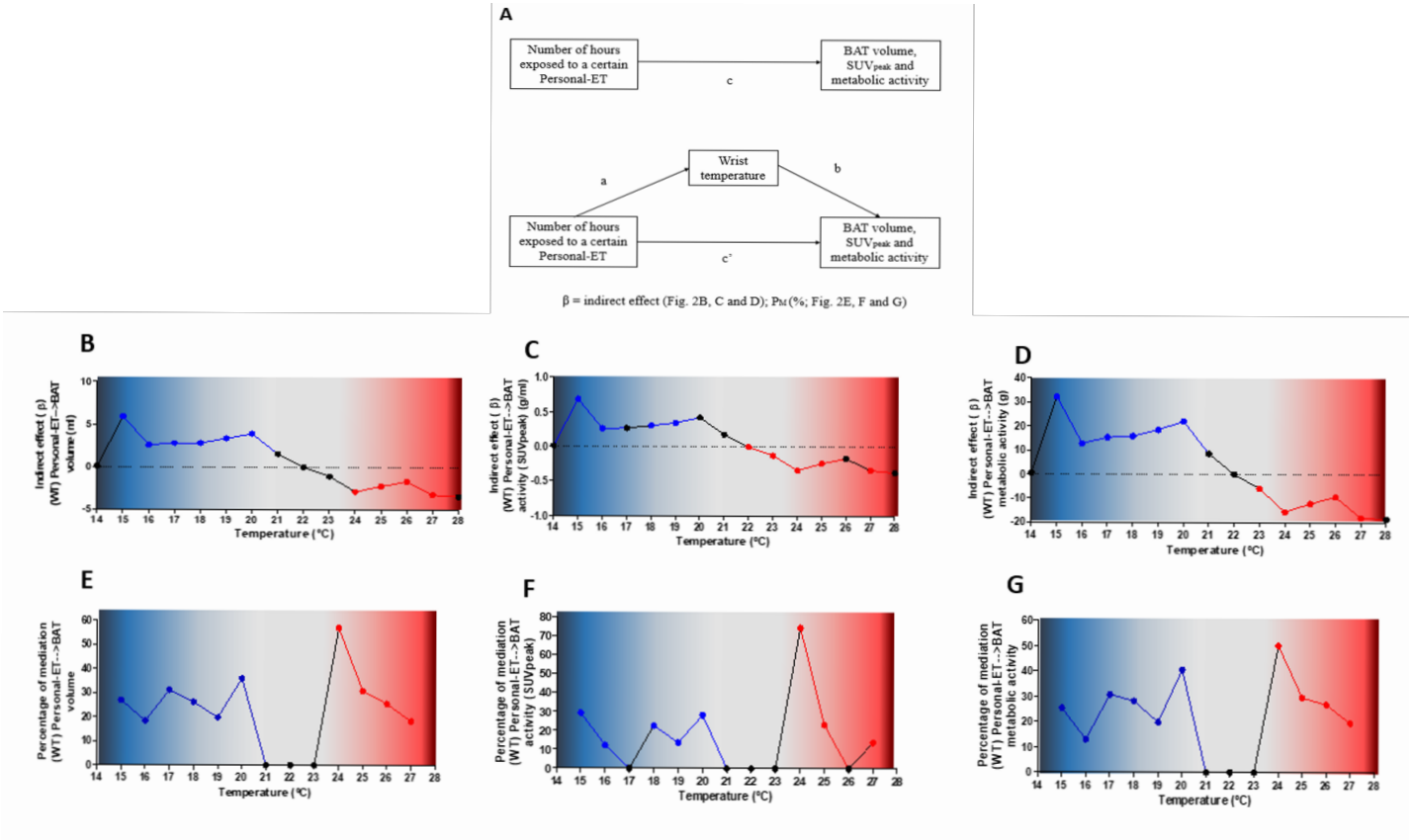

Figure 2. A) Mediation models of the relationship between the number of hours exposed to a certain Personal-ET and BAT-related outcomes in young adults. Path c shows the association between independent and dependent variables. Arrows a x b show the natural indirect effect (β) pathway, and c' shows the natural direct effect pathway. B) Indirect effects (β) of the simple mediation analyses of wrist temperature on the association between the number of hours exposed to each degree of Personal-ET (from 14°C to 28°C) and BAT volume, whereas panels C and D refer to BAT SUVpeak and metabolic activity, respectively. E) PM of the simple mediation analyses of wrist temperature on the association between the number of hours exposed to each degree of personal-ET (from 14°C to 28°C) and BAT volume, whereas panels F and G refer to BAT SUVpeak and metabolic activity, respectively. Black dots represent that 0 was in the 95% confidence interval of the indirect effect, and, therefore, the mediation was considered non-statistically significant (P>0.05). Red and blue dots mean that the mediation analysis was statistically significant but with a different direction. BAT: brown adipose tissue; Personal-ET: personal level of environmental temperature; WT: wrist skin temperature; PM: Percentage of mediation.

DISCUSSION

The present study quantifies, for the first time, the mediating role of human BAT and skeletal muscle cold-induced activity in the relationship between personal level of environmental temperature and human wrist temperature as an indirect proxy of skin blood flow. Intriguingly, the results show that BAT volume and metabolic activity mediate up to 25.4% of the association between Personal-ET and WT. Moreover, the results indicate that the association of the number of hours exposed to a certain Personal-ET with BAT volume, SUVpeak, and metabolic activity is mediated by WT at temperatures from 14°C to 20°C and from 24°C to 28°C, but not in the thermoneutral zone, as expected. We did not find a mediating role of human skeletal muscles or a relationship between WT and skeletal muscles. We also found that the participants with lower WT (inducing higher peripheral vasoconstriction) at the coldest temperatures had higher levels of BAT volume, SUVpeak and metabolic activity, whereas the participants with higher WT (inducing higher peripheral vasodilation) at the warmest temperatures had lower levels of BAT volume, SUVpeak and metabolic activity. These findings show how WT (as a proxy of blood flow) is related to BAT volume and activity (SUVpeak) in young adults. However, further studies are needed to elucidate the possible mechanisms behind these relationships.

The mediating role of BAT

We show that both BAT volume and metabolic activity have a mediating role in the relationship between Personal-ET and WT measured in daily living conditions independently of the sex, BMI, LMI and FMI. This indicates that participants with who were exposed to the same Personal-ET during the 7 day had different WT, which is explained, at least in part, by different levels of BAT volume or metabolic activity. Therefore, by every 1°C that the personal-ET is decreased, BAT volume would explain approximately an increase of 0.0194°C in WT. The relationship between Personal-ET and the WT daily pattern has been widely used in the field of chronobiology [17,33]. Several studies compared WT daily patterns in obese vs. normal-weight women [34], young vs. older men and women [19,20], and men vs. women (20), and showed worse patterns (higher variability and higher daytime values) of WT in obese and older participants. These findings are also in accordance with those of human BAT studies, which showed that obese, older people, and men have lower BAT volume and activity [35]. Therefore, we postulate that BAT volume and metabolic activity should be taken into account in further chronobiological studies using WT, especially in those studies, which only measured WT as a proxy of the circadian pattern without the inclusion of the Personal-ET. We established that based on the following facts: (i) the observed mediating role of human BAT volume (and metabolic activity) in the relationship between Personal-ET and WT, (ii) the activation of BAT in cold ambient-temperatures (Personal-ET≤20°C), (iii) that obese, older people, and men have lower BAT volume and activity as well as worse patterns of WT, and (iv) the circadian rhythms and, specifically core body temperature rhythms are all controlled by specific neural pathways in the anterior hypothalamus [1]. For instance, Martinez-Nicolas et al. [17] studied the mediation role of WT in the relationship between Personal-ET and mean arterial blood pressure in summer and winter, and postulated that BAT could mediate this relationship. In this study, we show that this hypothesis might be true, although further studies are needed to fully understand the possible mechanisms behind these assumptions.

The mediating role of WT

All the physiological mechanisms of the thermoregulatory system seem to be orchestrated in the preoptic area (POA) of the hypothalamus [1]. In addition to the peripheral tissues, the temperature of the brain is an input into the thermoregulatory system [36]. One of the hypotheses explaining why human BAT is placed in the cervical and supraclavicular zone is because, as a thermogenic tissue, its main function is

to regulate the temperature of the blood going to the brain [37,38]. Several studies have shown that human BAT activation is related to an increase in the blood flow in BAT [15,39]. Based on these results, the present study postulate that the increase in BAT activation (blood flow) could result in a redistribution of the blood in the peripheral part of the body during a cold stimulus which is moved into BAT in order to generate heat, since BAT is highly irrigated [40]. In contrast, during a warm ambient, the blood flow in the peripheral part of the body increases at the same time as BAT blood flow and activation decrease.

Warm

In warm-ambient environments (from 24°C to 28°C), we observed that the higher the Personal-ET is, the higher WT is, which is associated with a lower BAT volume and activity. Therefore, by every hour exposed at 27°C (personal-ET), WT would increase and explain approximately a decrease of 3.2 ml of BAT volume. The skin has warm-sensitive neurons specially to perceive the warm exposures [1]. However, there is some controversy as to which the main transient receptor potential (TRP) channel to be involved as a warm sensor is, the candidates being TRPV1, TPRV3, TPRV4, and TRPM2 [1]. Therefore, there might be participants with higher or more number of TRP channels than others, and this fact could explain why there are different responses to the same stimulus, although further studies are needed. Regardless of the main TRP channel involved, our results suggest that when Personal-ET is high (hot), the body initiates some physiological response in order to preserve core temperature. Thus, the main physiological mechanism involved is to induce a peripheral vasodilation with an inhibition of human BAT (redistribution of blood flow to peripheral regions to dissipate heat). We also showed that the higher the WT is, the lower the levels of BAT volume and activity (inhibition of this tissue) are. For instance, two participants that spent the same time in warm ambient, the participant with higher WT also had lower levels of BAT volume, which might indicate more efficiency adapting to warm temperatures, which reciprocally would implicated less efficient response to cold.

Cold

In cold-ambient exposures (from 14°C to 19°C), we showed that the lower the Personal-ET is, the lower WT is, which is associated with higher BAT volume and activity. Therefore, by every hour exposed at 15°C (personal-ET), WT would decrease and explain approximately an increase of 2.5 ml of BAT volume. In the skin of the peripheral parts of the body, there are also cold-sensitive neurons. These cold-sensitive neurons highly expressed levels of transient receptor potential cation channel subfamily M member 8 (TRMP8), which is the primary peripheral cold sensor in the thermoregulatory system [41]. Animal models have shown that the inhibition of this sensor inhibits the behavioral and physiological responses to cooling [41]. Taking this into consideration, we can postulate that there are individuals with a more efficient thermoregulatory system against cold stimuli, inducing a higher peripheral vasoconstriction and BAT activation in order to keep the core body temperature constant, which could be explained by a higher sympathetic tone. According to this, it might be possible for people with higher levels of human BAT to have a higher concentration of TRMP8, as well as different polymorphism of the gene TRMP8 might be associated with a better response to cold stimuli; however, these hypotheses have not been studied so far.

The mediating role of skeletal muscles

Skeletal muscles are involved in the thermoregulatory responses during cold exposure [6,11]. Interestingly, we did not observe an effect of the skeletal muscle activity (as measured by the 18F-FDG uptake) in the relationship between Personal-ET and WT. This lack of mediating effect does not necessarily mean that skeletal muscle is not involved in cold-induced thermogenesis. This lack of effect might be due to the fact that the

cold-ambient temperatures were not cold enough to induce skeletal muscle activation, or because the 18F-FDG tracer is not a good marker of skeletal muscle metabolism [6].

We postulate, however, that there are participants who respond better (i.e. responders) than others to cold exposures, and others that respond better to warm exposures, yet further studies are needed. This assumption is also based on the fact that some people could have an overexpression of POA neuron levels or TRMP8 or TRP channels [1], making the thermoregulatory system more efficient, or maybe in the brain the areas involved in the thermoregulatory system are different. This cross-sectional and observational study should be replicated in older participants and using other nuclear tracers such as 15O-O2, [11C]-acetate [6], or adenosine perfusion, a vasodilator that seems to active human BAT [28]. Moreover, we know that during sleep phases humans can lose at least 25% of their total thermoregulatory capacity. Since our aim was to study the mediating role of human BAT during 7 days (even in sleep phases) we keep these analyses as main results, although excluding the sleep phase did not alter the results (data not shown). Moreover, in this study the level of clothing during the measurements were not evaluated. Future experimental studies are warranted to elucidate the possible mechanisms behind this efficiency in the thermoregulatory system and new therapies that could be developed to improve this physiological system.

CONCLUSIONS

We show that BAT volume and metabolic activity mediate the relationship between Personal-ET and WT. Moreover, our data support that the individuals who were exposed to lower environmental temperatures and at the same time had lower wrist skin temperature, concomitantly had higher BAT volume. We also observed the opposite effect when the participants were exposed to warmer temperatures, which indicates a redistribution of the blood flow between the peripheral part of the body and BAT activation/inhibition in order to keep the core body temperature constant. Future interventional studies should try to find strategies to improve the thermoregulatory system and its relationship with metabolic diseases.

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Skin temperature response to a liquid meal intake is different in men than in women

CHAPTER 11

BACKGROUND

Obesity is considered a pandemic disease that has increased exponentially during the last decades [1]. A study conducted in 19.2 million participants from 200 countries indicated that, by 2025, global obesity prevalence will reach 18% in men and surpass 21% in women [1]. In simple terms, obesity is caused by an energy imbalance where energy intake exceeds the total energy expenditure resulting in an increase in energy storage in the body as fat mass [2]. Thus, new insights on the regulation of energy balance regulation can provide additional information about the development of obesity and possible treatments to face it [3].

In thermoneutral conditions, the total energy expenditure can be divided into resting metabolic rate (RMR), activity energy expenditure (AEE), and diet-induced thermogenesis (DIT) [4]. The latter represents between 7 and 12% of the total energy expenditure [5]. DIT is the energy expenditure required for the digestion, absorption, and storage of food that derives energy throughout a day [2]. Meal-induced thermogenesis or the thermic effect of food (TEF) refers to the increase of the metabolic rate and body temperature [6] in response to a single meal, and it lasts from 3 to 6 hours after the meal intake [2,6].

The TEF is influenced by the macronutrient composition of the diet[7–9] as well as by the form of the meal (i.e. solid or liquid) [10,11]. The TEF is higher after a liquid meal intake [10] than after a solid meal intake [11] while keeping the same nutritional composition. Liquids, which are emptied from the stomach faster than solids, are, therefore, expected to be absorbed more quickly [10,12].

The digestion and absorption of nutrients increases body temperature, yet the physiological responses regulating the process are still unclear [6]. Moreover, whether the thermic effect of a liquid meal intake is different in men than in women remains to be investigated. In the 90's, Westerterp-Plantenga et al. [13] reported an increase of the skin temperature in women after a solid meal. It is known that women feel a higher thermal discomfort than men, whereas, in men, the skin temperature of their limbs seems to be slightly lower in thermoneutral conditions [14]. This could be partially explained because women have less blood flow in their hands [15] while men's core temperature [16] seems to be higher [14,17].

To date, most of the studies have focused to determine the TEF in terms of energy expenditure, but little is known about which is the response in terms of skin temperature. Therefore, the aim of the current study was to determine whether the TEF with a standardized and individualized liquid meal on skin temperature is different in young adult men than in young adult women.

MATERIAL AND METHODS

Participants

A total of 104 (36 men and 68 women) white Caucasian healthy adults aged 18-25 years old, and with a body mass index (BMI) range of 17.5-33.7 kg/m2, participated in the present study. The participants were enrolled in the ACTIBATE study [18], an exercise-based randomized controlled trial (Clinical Trials.gov ID: NCT02365129). All participants were non-smokers, were not enrolled in a weight loss program, had a stable body weight (body weight changes <3 kg) over the last 3 months, were not physically active (<20 minutes on <3 days/week), did not take any medication, had no acute or chronic illness, and were not pregnant. The study was conducted between October and November in 2015 and 2016 in Granada (Southern Spain). The study protocol and informed consent were performed in accordance with the Declaration of Helsinki (revision of 2013), and they were approved by the Human Research Ethics Committee of both University of Granada (n° 924) and Servicio Andaluz de Salud (Centro de Granada, CEI-Granada). A written informed consent was obtained from all the participants.

Design

The measurements started between 8.30 and 9.30 AM. The participants arrived at the

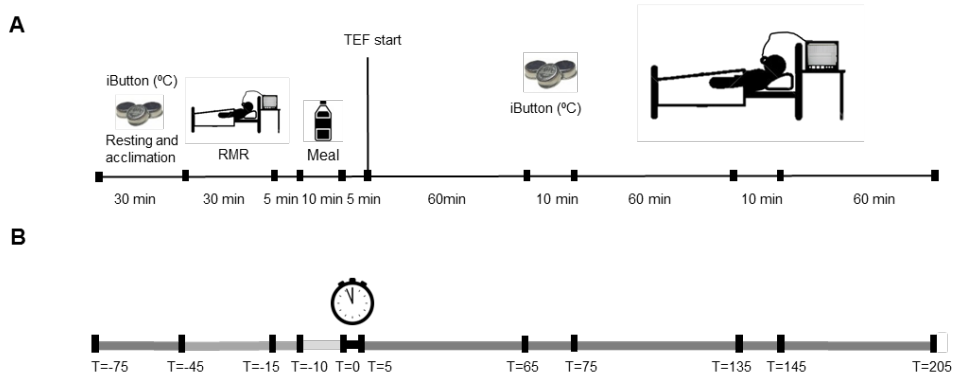

Figure 1. Thermic effect of feeding (TEF) test. A: Procedures; B: Accumulative timeline. RMR: Resting metabolic rate.

research center by car or bus, avoiding any physical activity after waking up, and in a fasted state (12 hours after their last meal). The night before taking the measurements, the participants had a standardized meal (tomato sauce with boiled rice and a one-egg omelet). Moreover, prior to the study day, they were instructed to refrain from drinking alcoholic or caffeine-containing beverages (24 hours before the testing day), to not do any moderate or vigorous physical activity (48 hours before the testing day), as well as to sleep as usual. The tests were carried out in a quiet room with dim lighting. The ambient temperature and humidity were 22.9±0.7°C and 44.6±6.3%, respectively. During the testing day, the participants lay down on a reclined bed, on a supine position, and were covered with a sheet. They wore standardized clothes (shorts, standardized T-shirt, and were barefoot, Clo value=0.20 [19]). Before starting the measurements, the women reported their menstrual cycle phase (menstrual, follicular, ovulatory, or luteal phases). The participants were instructed to breathe normally, and not to talk, fidget, cross their arms or legs, or sleep.

Resting and acclimation period: At the beginning of the testing day, the participants entered the room, the above-mentioned conditions were checked, and the iButtons were attached [20]. Then, an acclimation and a resting period were performed in order to obtain more stable measurements of the RMR and skin temperatures (see Figure 1 A and B, respectively).

Baseline period: The RMR and skin temperatures were measured during 30 minutes. The RMR was measured by indirect calorimetry (IC) (Ultima CardiO2, MGU, Medgraphics Corp, Minnesota, USA; and CCM Express, CCM)[21]. The participants wore a neoprene face-mask without external ventilation or face-tent mask depending on the metabolic cart used. We performed calibrations following the manufacturer's instructions before every IC measurement. The last five minutes of the baseline period were averaged for further analyses.

TEF period: Once the participants had completed the liquid meal test, the skin temperature parameters were registered during 3 h and 20 min. The participants had a 10-minute 'rest' every hour (also referred to as period), resulting in three periods of one hour. During these breaks, the participants were allowed to sit up, go to the toilet, or drink water, if needed. Moreover, they reported the thermal sensation of the whole body, feet, clavicular, and hands zones using visual analog scales (VAS). The VAS consisted in a line of 100 mm in length with words anchored at each end (0mm = "No cold at all", 100mm = "Maximum tolerable cold")[22].

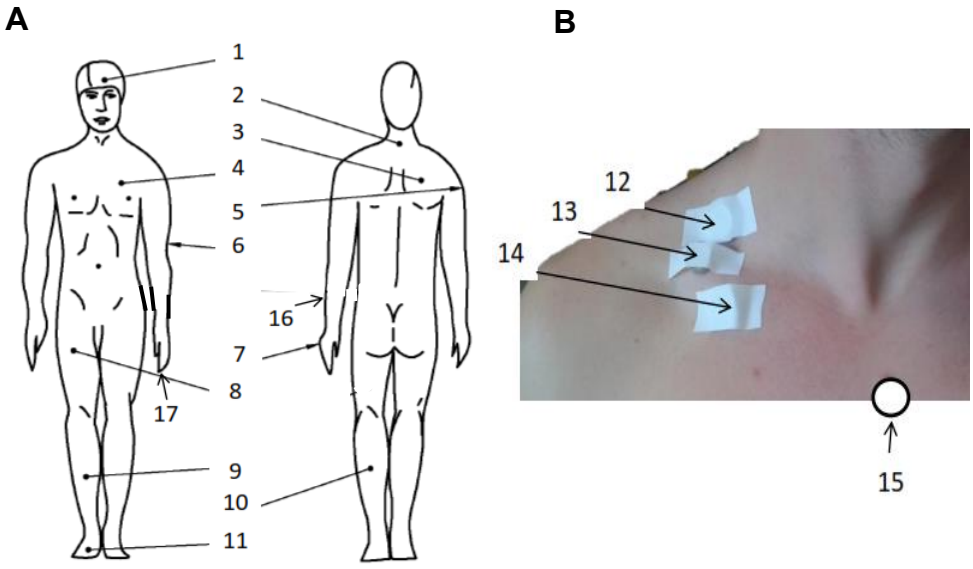

Figure 2. Anatomical position of 17 iButtons. A: Distribution of the iButtons over the body. B: Distribution of the iButtons on the right clavicular site [20].

Meal test

After the baseline measurements, the participants consumed a standardized and individualized liquid meal refrigerated at approximately 4 °C. The amount of the liquid meal was adjusted to 50% of each participant's RMR (i.e.: 1583 ± 498 kcal and 1288 ± 423, men and women respectively, see Table 1). The meal test (T- Diet Energy, Vegenat ®) had a density of 1.186 g/ml and a caloric density of 1.6 kcal/ml. The percentage of the energy derived from the nutrients was as follows: 15% proteins, 47% carbohydrates, and 35% fats, of which 25% were saturated,

Table 2. Equations to measure the skin temperature.

Outcome	iButtons (n)	Anatomical positions. Figure 2	Equation	Reference
Mean skin temperature	8	1,3,4,5,6,7,8, 10	(Forehead*0.07)+(Right Scapula*0.175)+(Left Chest*0.175)+(Right Deltoid*0.07)+(Left Elbow*0.07)+(Left Hand*0.05)+(Right Thigh*0.19)+(Left Gastrocnemius*0.2)	8 ISO 9886-2004 [30]
Proximal skin temperature	3	3,4,15	(Left Chest+Right Scapula+Upper Breastbone)/3	Martinez-Tellez et al. [20]
Distal skin temperature	2	7, 11	(Left Hand+Right Instep)/2	Kräuchi et al. [31]
Supraclavicular	1	12	Right Supraclavicular	Boon et al. [32]
Peripheral temperature Gradient	2	16, 17	(Left Forearm-Left Top of forefinger)	Adapted to Sessler et al. [33]

Table 1. Descriptive characteristics of the study participants.

	Men (n=36)			Women (n=68)			P
Age (years)	22	±	2.2	21	±	2.1	0.174
BMI (kg/m2)	27	±	5.7	23	±	3.7	**0.001**
RMR (kcal/day)	1583	±	498.2	1288	±	423.8	**0.002**
Liquid meal (g)	494	±	155.7	402	±	132.4	**0.002**
Fat mass (kg)	25	±	11.9	24	±	7.5	0.526
FMI (kg fat mass/m^2)	8	±	3.8	9	±	2.6	0.393
Fat mass (%)	30	±	7.7	38	±	5.9	**< 0.001**
Lean mass (kg)	53	±	7.0	36	±	4.9	**< 0.001**
LMI (kg lean mass/m^2)	17	±	2.0	13	±	1.3	**< 0.001**
Lean mass (%)	65	±	7.1	57	±	5.4	**< 0.001**

Data are mean ± standard deviation. BMI: Body mass index, RMR: Resting metabolic rate, FMI: Fat mass index, LMI: Lean mass index

50% monounsaturated, and 25% polyunsaturated, and with a content of 3% of fiber.

Procedures

Skin temperature and equations

We measured the skin temperature with 17 iButtons [20] (DS-1922 L, Thermochron; resolution: 0.0625°C; Maxim, Dallas, USA), which are valid and reliable devices to measure skin temperature in humans [23,24]. We attached the iButtons to the skin with adhesive tape (Fixomull, Beiersdorf AG, Hamburg, Germany) at different body sites (see Figure 2) [23,25–28]. We recorded the skin temperature at 1-minute intervals, and we calculated the averages for every 5 minutes in order to analyze the data [20]. The mean, proximal, distal, and supraclavicular skin temperature parameters were estimated (see equations in Table 2). Moreover, a peripheral gradient was calculated as a proxy of a peripheral vasoconstriction (forearm-fingertip) [29]. All data registered by the devices and equations were analyzed by the Temperatus® software [20] (http://profith.ugr.es/temperatus). The measurements of the skin temperatures during the first 30 minutes of each period were excluded from the analyses.

Figure 2. Anatomical position of 17 iButtons. A: Distribution of the iButtons over the body. B: Distribution of the iButtons on the right clavicular site [20].

Body composition

The body composition was measured by Dual Energy X-ray Absorptiometry (DEXA) scan (HOLOGIC, Discovery Wi). We measured the participants' weight and height without shoes and wearing the standard clothes using a SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany), and we calculated the BMI (kg/m2). The fat mass index (FMI) was calculated as kg of body fat divided by height in m2. Similarly, we calculated the lean mass index (LMI) as lean body mass in kg divided by height in m2.

Statistical analysis

The data are presented as mean and standard deviation, unless otherwise stated. Sex differences were determined using the T-test for independent samples. We conducted an analysis of variance (ANOVA) for repeated measurements with Bonferroni adjustments for post-hoc comparisons to compare the 5-minute average of the skin temperature in response to the consumption of the liquid meal with the 5-minute average of the baseline period (before the intake). The analyses were conducted by periods (baseline, first hour, second hour, and third hour). We calculated the area under the curve (AUC) for each period using the trapezoidal rule for the VAS and as a percentage of increase of the baseline period for the skin temperature. The total AUC was calculated as the weighted sum of the AUC of the three periods of the TEF. To compare the thermal perception of each period with the basal thermal perception, we conducted ANOVA for repeated measurements with Bonferroni adjustments for post-hoc comparisons. We repeated all the analyses including BMI, FMI, LMI or menstrual cycle in the model as co-variable. All analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set at <0.05.

RESULTS

Characteristics of participants

Table 1 shows the participants' characteristics. Women had lower BMI and RMR than men (P<0.01), and men were leaner and had lower levels of fat mas than women (all P<0.01).

Mean, proximal, and distal skin temperatures

We observed a significant increase of the mean skin temperature after the liquid meal intake had peaked in men in the 2nd h (Figure 3A) and in women in the 3rd h (Figure 3B). Overall, women had a higher increase of the mean skin temperature after the liquid meal intake than men (P≤0.01, Figure 3C).

Figure 3D and 3E show the TEF on the proximal skin temperature in men and women, respectively. There was a significant increase after the liquid meal intake in all measurement points, which was similar in both men and women [Figure 3F, (1st h: P=0.958; 2nd h: P=0.175; 3rd h: P=0.206; and Total period: P=0.279)].

Figure 3G shows the TEF on the distal skin temperature in men. There was a significant decrease between the 35th and 40th minute of the 1st h. In women, the distal skin temperature significantly decreased after the liquid meal intake (1st h, all P≤0.05), yet it significantly increased at the 175th minute and continued increasing until the end of the test (Figure 3H). In men we did not find any significant increase (Figure 3G). Sex differences were observed in the 3rd h after the liquid meal intake, being higher in women than men (P=0.012, Figure 3I).

Supraclavicular skin temperature

Figure 3J shows the TEF on the supraclavicular skin temperature in men, which was constant throughout the test. In women, there was a significant increase of the supraclavicular skin temperature in the 2nd hour, as well as in the last 10 minutes of the 3rd hour (all P≤0.05, Figure 3K). Women had a significantly higher supraclavicular skin temperature after the liquid meal intake in the 1st h than men (P=0.027, Figure 3L).

Peripheral gradient as a proxy of peripheral vasoconstriction

Figure 3M shows the TEF on the peripheral gradient in men, which did not change during the test. In women, the peripheral vasoconstriction significantly increased from the 35th to the 45th minute in the 1st h (all P≤0.05, Figure 3N). However, we did not find any significant difference in the 2nd h compared with the baseline measurement (all P>0.05). In the 3rd h, the peripheral vasoconstriction significantly decreased (P≤0.05).

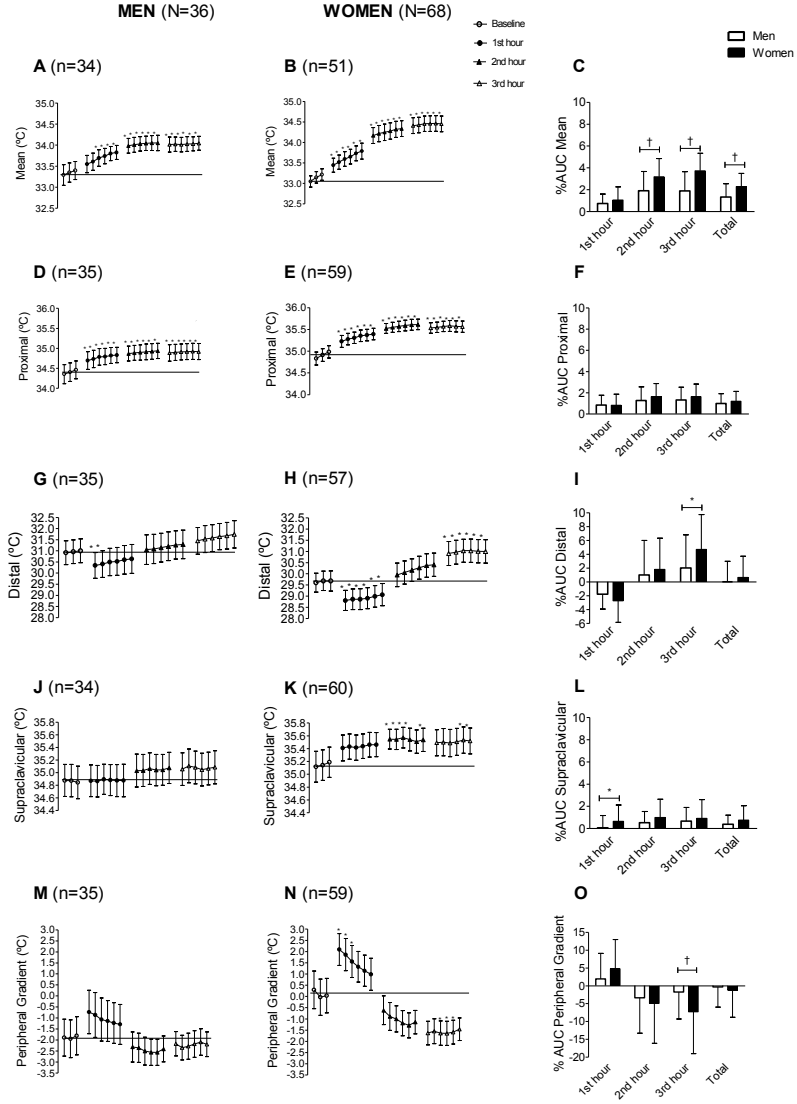

Figure 3. Kinetics of skin temperature parameters. The graphic bars represent sex differences expressed as area under the curve (AUC). The measurements of the skin temperatures during the first 30 minutes of each period were excluded from the analyses. Kinetics of the skin temperature are presented as the mean of every 5 minutes and upper and lower limits of the interval of confidence (95%). Analysis of variance with Bonferroni comparisons regarding the last five minutes of the baseline period. A., D., G., J., and M. refer to the mean, proximal, distal, supraclavicular, and peripheral gradient (as a proxy of the peripheral vasoconstriction) temperatures in men, respectively. B., D., H., K., and N. refer to the mean, proximal, distal, supraclavicular, and peripheral gradient (as a proxy of the peripheral vasoconstriction) temperatures in women, respectively. Open circles: Baseline defined as the last fifteen minutes of measurement before the intake of the standardized individualized liquid meal. Black circles: First hour in response to the thermic effect of feeding (1st hour). Black triangles: Second hour in response to the thermic effect of feeding (2nd hour). Open triangles: Third hour in response to the thermic effect of feeding (3rd hour). C., F., I., L., and O. refer to percentage of increase respect to the baseline in terms of area under the curve for each period: First hour in response to the thermogenic effect of feeding. 2nd hour: Second hour in response to the thermogenic effect of feeding. 3rd: Third hour in response to the thermogenic effect of feeding. Total: It is the weighted sum of the 3 hours in response to the thermogenic effect of feeding in the mean, proximal, distal, and supraclavicular skin temperature, and the peripheral gradient, respectively. The parallel bars indicate significant sex differences. * P≤0.05, † P≤0.001.

Thermal perception: VAS measurements

Figure 4A shows the TEF on the whole body thermal perception in men. Men felt less cold in the 2nd and 3rd h (P=0.029 and P≤0.01, respectively). Women showed a similar pattern to men in the same hours (P≤0.01) see Figure 4B. Regarding to the thermal perception in the clavicular zone, we did not find any change over the test in either men or women (Figure 4C and 4D, respectively).

Figure 4E and G shows the TEF on thermal perception in the feet and hands zone in men, who felt less cold after the 2nd and 3rd h with respect to baseline. Moreover, we observed a similar trend in women's feet and hands (Fig. 4F and 4H, respectively). However, women felt their hands colder after the intake (P≤0.01), and they felt their feet and hands less cold after the 2nd (P≤0.01) and 3rd h (P≤0.01), whereas men did not feel any cold during the test.

All of the analyses were repeated adjusting by BMI, FMI, or LMI, and the results persisted (all P for trend ≤0.05). Furthermore, there were no differences in the TEF or the thermal sensation by menstrual phases (follicular, n=19; ovulation, n=5; luteal, n=28; and menstrual, n=9) (all P for trend ≤0.05). We conducted linear regression analyses with the AUC of parameters of skin temperature (i.e. mean, proximal, distal, etc.), and the AUC of thermal perceptions (body, clavicular, feet and hands). We did not find any significant relationship (Figure 5). The results persisted when the sample was divided into BMI categories and sex (all P ≤0.05).

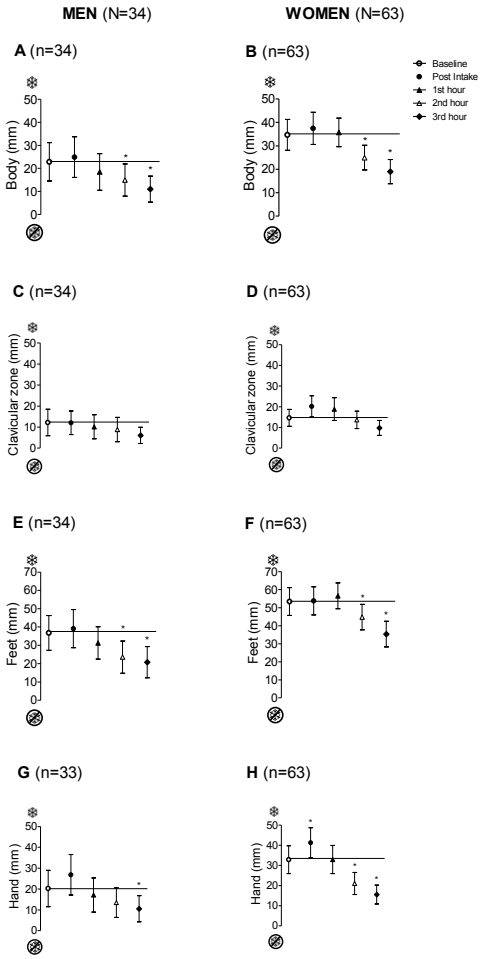

Figure 4. Kinetics of thermal perception: VAS measurements. The sex differences are represented as graphic bars between the areas under the curve (AUC). Kinetics of thermal perception is presented as the mean of every period and upper and lower limits of the interval of confidence (95%). Analysis of variance with Bonferroni comparisons in relation to the baseline period. A., C., E., and G. refer to the body, clavicular area, feet, and hand thermal perceptions in men, respectively. B., D., F., and H. refer to the body, clavicular area, feet, and hand thermal perceptions in women, respectively. Open circles: Baseline defined as the period before the intake of the standardized individualized liquid meal. Black square: Post intake, this is the moment just after the intake of meal. Black triangles: First hour in response to the thermic effect of feeding (1st hour). Open triangles: Second hour in response to the thermic effect of feeding (2nd hour). Black diamond: Third hour in response to the thermic effect of feeding (3rd hour). In the upper extreme of axis "y", refers to "maximum tolerable cold", and in the lower extreme of axis "y", represents "do not feel any cold".

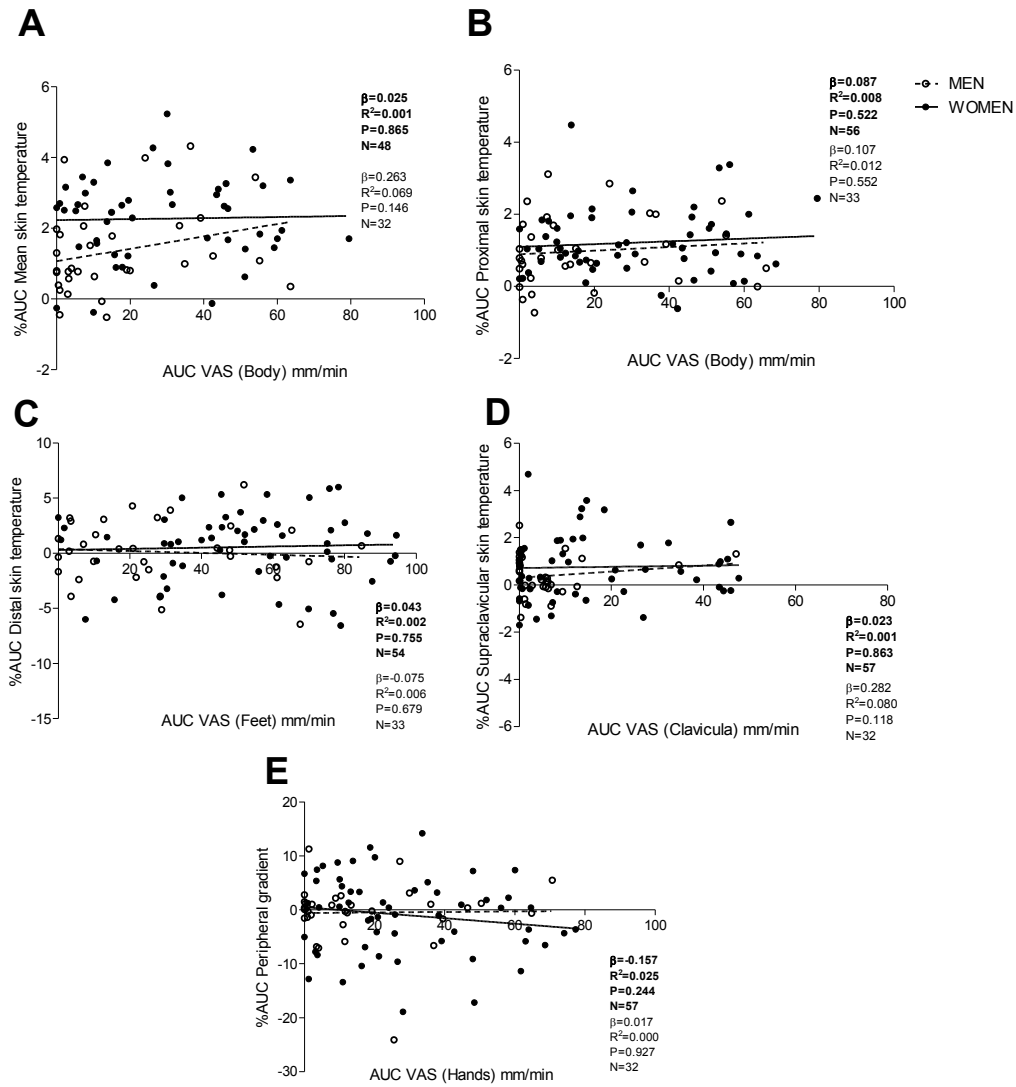

Figure 5. Linear regression between the area under the curve (AUC) of thermal perception in different zones with the AUC of the parameters of skin temperature at the end of the test. Black circles refer to women, whereas open circles refer to men. A., linear regression between the AUC of the visual analog scale (VAS) referring to the body thermal comfort with mean skin temperature. B., linear regression between the AUC of the VAS referring to the body thermal comfort with proximal skin temperature. C., linear regression between the AUC of the VAS referring to feet thermal comfort with distal skin temperature. D., linear regression between the AUC of the VAS referring to the clavicular thermal comfort with supraclavicular skin temperature. E., linear regression between the AUC of the VAS referring to hands thermal comfort with peripheral vasoconstriction gradient.

DISCUSSION

The present study shows an increase of the mean, proximal, and supraclavicular skin temperature after the intake of a standardized and individualized liquid meal test in young adults, being this thermoregulatory effect higher in women than in men. We also observed that the distal skin temperature decreased during the first hour and then increased during the second and third hour in both, being significant in women. In addition, there was a postprandial

peripheral vasoconstriction during the first hour and a later vasodilatation during the second and third hour only in women. Overall, the TEF was higher in women than in men in all skin temperature measurements, except in the proximal skin temperature, which was similar in men and in women. Women always felt colder than men, especially in the first postprandial hour. Of note is that these findings persist after controlling for body composition and were independent of the menstrual phases. To date, most published studies have focused on the increase of energy expenditure after a meal intake, and little is known about which is the response of the skin body temperature after a meal intake. Therefore, the findings of the present study shed light on this topic and show that temperature response after a liquid meal intake is different in men than in women.

Components of thermic effect of feeding (TEF)

It is well known that the TEF stimulates an increase in metabolic rate which is accompanied by an increase of the body temperature [6]. This increase in metabolic rate in response to a meal seems to be regulated by the parasympathetic nervous system, among others, but it is unknown if the autonomic nervous system regulates the increase of body temperature[6]. Regarding the increase in the metabolic rate, it seems that it is higher in men (21%) in comparison to women after the intake of different meals, even adjusting by age, BMI, or waist circumference [34]. Davidson et al. [35] reported that the sympatho-adrenal activity was higher in men than women. Vaz et al. [36], on the other hand, found that the increase of energy expenditure was linked to postprandial sympatho-adrenal activity, albeit this relationship seems to be attenuated because women had less sympathetic nervous system (SNS) activity, regardless of the body composition.

Thermoregulatory system: skin temperature

The thermoregulatory system seems to be regulated by the SNS[37]. In our study, women experienced a higher increase of the skin temperature parameters than men. Moreover in our sample, body composition did not explain these sex differences, which are in agreement with others [34]. Of note is that the SNS activity after food intake is lower in women than men [35]. Why does this phenomenon occur?

The thermoregulatory system is mainly controlled by the preoptic anterior hypothalamus (POA), the homeostatic control of which is separated from the sensation of temperature [38,39]. It is well known that human skin has cold and warm-sensitive POA neurons as peripheral receptors. However, all thermoregulatory responses could be triggered by either the activation of one class of the temperature-sensitive neurons (cold vs. warm) or the inhibition of the others. Previous studies showed that different stimulus may change the activity of the cold or warm-sensitive POA neurons [38,40]. Studies in animal models observed that females [41] have a larger amount of warm than cold-sensitive POA neurons in comparison to males. Vries & Södersten [38] proposed that the nucleus and POA was bigger in men than in women, and that the suprachiasmatic nucleus, which is involved in the reproduction cycle, was larger in women than in men [42]. Therefore, it is biologically plausible that the size of the different parts of the hypothalamus could modulate the different physiological responses. This may partially explain why the skin temperature response to a liquid meal is higher in women than in men. However, if women have more warm-sensitive POA neurons than men and whether it is associated with the size of the different parts of the hypothalamus is not known.

Moreover, there are other sex differences that could explain the differences between the thermal responses after a meal intake. For instance, the cutaneous vascular anatomy is different in women than in men [14]. Women seem to have less

vascularization in the distal parts of the body and therefore have less blood flow in their hands [15]. The thermoregulatory system goal is to keep the core temperature constant, being more important in women than men because their bodies are prepared to shelter life[43]. To our knowledge, there is only one study which focused on the increase of skin temperature after a meal intake, but the aim was not to study sex differences [13].

Body composition may have affected the measurement of the skin temperature by iButtons, because of its insulation effect [44], yet we found no body composition effect over the skin temperature response after a meal intake. This finding could be driven by the fact that in our experiment design we individualized the meal intake to the RMR, which indeed is body composition dependent. Therefore, we have indirectly adjusted the meal intake to the individual's body composition, and this may partially explain why we did not find any significant differences in terms of skin temperature by BMI.

Several studies suggested that brown adipose tissue (BAT) plays a role in DIT [45,46], yet the results are contradictory [47,48]. We observed slight changes in the supraclavicular skin temperature after a liquid meal intake in women. The supraclavicular skin temperature has been used as an indirect marker of BAT volume and activity [25,32]. We do not know, however, whether the supraclavicular skin temperature response to a liquid meal represents BAT activity. More studies with objective measurements of BAT are needed to understand the role of BAT in the thermoregulatory response to a meal intake.

Peripheral Thermoreception

A recent study investigated the molecular mechanisms of the cutaneous thermoreception suggesting that the transient receptor potential (TRP) family mediates thermal sensation across a broad physiological range of skin temperatures [49]. Nevertheless, none of these TRP channels have been identified as a molecular thermoreceptor, and we do not know if the prevalence of these TRP channels differ by sex, as occurs in other species [50]. Therefore, it would be reasonable to find different amounts of the TRP family in males and females, which could modulate the different thermal sensations [39]; unfortunately, there are no previous studies in humans.

Controversy exists on whether the skin temperature responses to a liquid meal are related to the thermal sensations in humans [51]. Thermal sensation in women is different than in men. Karjalainen [14] reported that women express more dissatisfaction than men in the same thermal environments, whereas in neutral temperature these differences disappear. They also reported that women are less satisfied than men in cooler conditions. Similarly, Schellen et al. [24] showed that women feel more uncomfortable and dissatisfied than men under the same cold conditions. Furthermore, they reported that the local sensation and skin temperature of the extremities (hands and arms) are of high importance for whole body thermal sensation, whereas this is less important in men. These findings concur with our results.

Limitations

The present study has several limitations that should be highlighted. It is known that the skin temperature follows a diurnal biorhythm [26,52], but the most stable skin temperature period is between 10 AM and 2 PM [53], the period when our experiments were conducted. The room temperature was slightly low, and therefore it will be of interest repeat the experiment with a higher room temperature. We have no control group; our control condition was the baseline period. Moreover, more studies are needed to understand the differences in terms of increase of the skin temperature after a meal intake between sexes, including participants with the same BMI, albeit it seems that body composition did not influence the skin temperature response in the present study.

CONCLUSIONS

Our findings show that a standardized and individualized liquid meal intake increases the skin temperature in young adults. Women presented a higher increase of skin temperature parameters than men in response to a meal intake, regardless of their body composition and menstrual cycle. Furthermore, our data suggest that the thermoregulatory system is more effective in women than in men: Women are able to reduce the temperature of the distal zones faster during the first hour (distributing the blood flow to the digestive system), and they are able to produce a higher and faster vasodilatation (distributing blood to the distal areas to carry the nutrients) without any alteration in the mean and proximal skin temperature.

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PART 4

Clinical Implication

Evidence of high 18F-Fluorodeoxyglucose uptake in the subcutaneous adipose tissue of the dorsocervical area in young adults

CHAPTER 12

BACKGROUND

Brown adipose tissue (BAT) is a thermogenic tissue with the ability to dissipate energy in the form of heat by oxidation of glucose and fatty acids, due to the unique presence of the uncoupling protein-1 (UCP-1) [1]. A potential clinical implication of activating BAT relates to its high metabolic activity and its potential role in stimulating energy expenditure, which makes it an attractive target to reduce adiposity [2].

In 2009, several studies using 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography (PET) combined with X-ray computed tomography (CT) (18F-FDG-PET/CT) imaging showed the presence of active BAT in adults upon cold exposure [3]. BAT depots were specifically identified mainly in the laterocervical and supraclavicular [4]. Collection of biopsies at this site is severely hampered by the deep and heterogeneous location of these depots, and most importantly, because of health risks [5]. Thus, identification of easily accessible BAT depots for biopsy purposes will help to better understand the role of this tissue in human energy metabolism and its relationship with adiposity.

Newborns corpses have subcutaneous BAT in the dorsocervical area [6] and, the existence of subcutaneous BAT in life-adults remained to be determined. Hence, the aim of the present study were: (i) to whether the 18F-FDG uptake in the SAT of the dorsocervical area is higher in comparison to the SAT of the other area, and (ii) to study whether the glucose uptake by SAT of these zones correlate with the glucose uptake of supraclavicular BAT, in young adults.

MATERIAL AND METHODS

A total of 133 sedentary healthy adults (age: 22±2 years; BMI: 25±5 kg/m^2) were included in this study. All participants were part of the ACTIBATE study and signed an informed consent [7]. The study was approved by the Ethics Committee on Human Research of the University of Granada (n° 924) and of the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada).

Personalized cold exposure

We introduced participants to a mild cold room (~19°C), dressed with a cooling vest with water perfused tubes. We gradually decreased the water temperature until the occurrence of shivering [8]. Two or three days later, we conducted a personalized cooling protocol during 2 hours [8]. At the end of this period we conducted the PET/CT scan (Siemens Biograph 16 PET/CT, Siemens, Germany) [8].

PET/CT analysis

BAT was quantified strictly following the international recommendations [9]. We quantified BAT in the classical human depots [4] following a protocol with high inter-observer reliability [10]. Moreover, we draw a region of interest (ROI) in the SAT of the dorsocervical (Fig. 1A) and tricipital area in order to quantify the 18F-FDG uptake at this area.

Infrared thermography experiments: confirmatory study

We took pictures with an infrared thermograph (FLIR b60 Systems) before and after personalized cold exposure in a separate sample of 11 adults (n= 10 women; age: 22±2 years, BMI: 22.5±3.1 kg/m^2) in order to study the skin temperature of the dorsocervical and supraclavicular area [11]. We draw a ROI in the supraclavicular, dorsocervical and abdominal areas and we took the maximal skin temperature inside every single ROI to perform the analyses.

Statistical analyses

We performed an unpaired t-test to study the differences in 18F-FDG uptake of the SAT differences between the dorsocervical vs. tricipital area. To test the effect of personalised cold exposure on skin

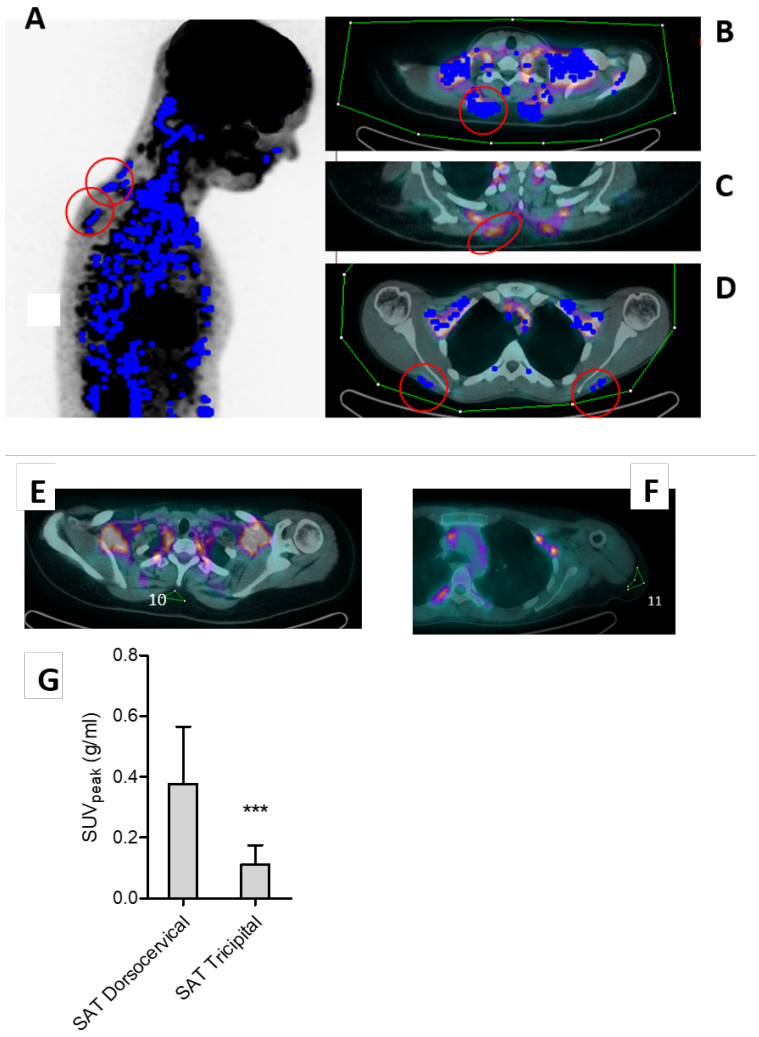

Figure 1. Example of a participant with 18F-fluorodeoxyglucose uptake by brown adipose tissue (blue dots) meeting the criteria of the Hounsfield units and standardized uptake value thresholds. Red circles represent 18F-fluorodeoxyglucose uptake by interscapular brown adipose tissue in the subcutaneous adipose tissue of the dorsocervical area (A-C) and in the subcutaneous adipose tissue behind both scapulas (D). E) Region of interest (ROI) drawn in the subcutaneous adipose tissue (SAT) of the dorsocervical area, around thoracic vertebrae 2. F) ROI drawn in the SAT of the tricipital area (as a control tissue). G) Differences between 18F-Flurodeoxyglucose (18F-FDG) uptake in the SAT of the dorsocervical area agains tricipital area. *** denote significant differences (P<0.001). Data are means and standard deviation.

temperature (i.e. supraclavicular, dorsocervical and abdominal) we performed paired t-test. Finally, we conducted linear regression analysis to study the association of 18F-FDG uptake of the SAT at the dorsocervical and tricipital area with supraclavicular BAT volume and activity. All analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM SPSS Statistics, IBM Corporation) and the level of significance was set to ≤0.05.

RESULTS

Firstly, we found that 18F-FDG uptake in the SAT of the dorsocervical area (Fig 1E) was significantly higher in comparison to SAT of the tricipital area (i.e. triceps; Fig 1F) (P<0.001) in all participants (n=133; Fig. 1G). Moreover,

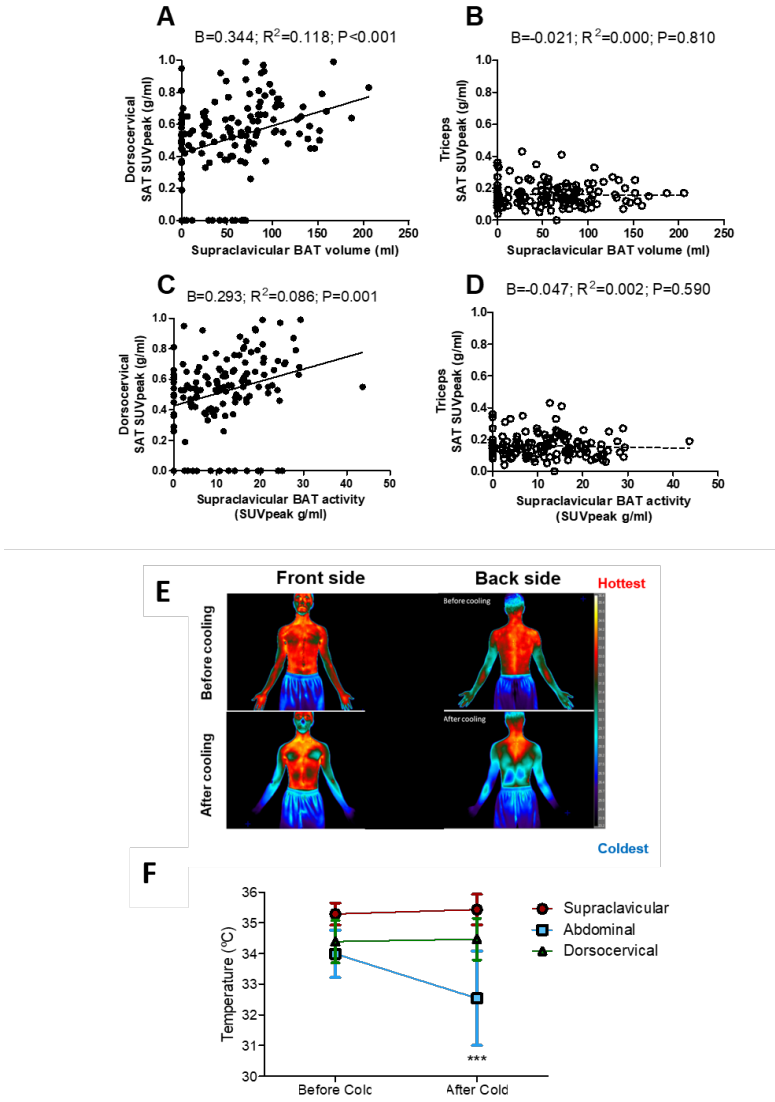

Figure 2. Linear regression between 18F-fluorodeoxyglucose (18F-FDG) uptake [standardized uptake value (SUVpeak)] measured in the subcutaneous adipose tissue (SAT) at the dorsocervical and tricipital area with supraclavicular brown adipose tissue (BAT) volume and activity (SUVpeak). Correlation analysis was performed between A) 18F-FDG uptake by SAT of the dorsocervical area activity (SUVpeak) with supraclavicular BAT volume. B) 18F-FDG uptake by SAT of the tricipital area activity (SUVpeak) with classical BAT volume. C) 18F-FDG uptake by SAT of the dorsocervical area activity (SUVpeak) with supraclavicular BAT activity (SUVpeak), and D) 18F-FDG uptake by SAT of the tricipital area activity (SUVpeak) with supraclavicular BAT activity (SUVpeak). B= standardized coefficient, R= regression coefficient. E) Infrared thermography picture of a representative participant before and after an individualized cold exposure protocol. F) Maximal skin temperature in the supraclavicular area, abdominal area (front) and dorsocervical are (back), before and after 2 hours of personalized cold exposure (n=11). *** Before vs. after cold exposure (P<0.001). Data are means and standard deviation.

we found that the 18F-FDG uptake by SAT of the dorsocervical area correlated with the total volume of supraclavicular BAT depots (Fig. 2A) and with the activity of those depots (SUVpeak, Fig. 2C). However, no associations were observed between 18F-FDG uptake by

SAT of the tricipital area and supraclavicular BAT-outcomes (Fig. 2B and D). Even the results persisted when SUVpeak was normalized by lean body mass [4] (data not shown).

During the initial analysis of human BAT in participants of the ACTIBATE study, we noticed that several participants had high 18F-FDG uptake in the SAT of the dorsocervical area, which achieved the recommendations to be quantified as BAT (Fig. 1A,B,C and D). We categorized participants as dorso-PET+ (i.e. 18F-FDG uptake in the SAT that achieved the recommendations to be considered BAT), and all other participants as dorso-PET- (i.e. 18F-FDG uptake in the SAT that did not achieve the recommendations to be considered BAT). The dorso-PET+ group (n=23) tended to be younger (21±2 vs. 22±2 years old, P=0.091 from unpaired t-test), and had lower BMI (23.2±3.9 vs. 25.3±4.8 kg/m^2, P=0.043 from unpaired t-test) compared with the dorso-PET- group (n=110). Moreover, the vast majority of the dorso-PET+ individuals were women (96%; n=22/23). Next, we conducted a confirmatory study to evaluate the relevance of the SAT of the dorsocervical area for temperature maintenance after cold exposure. We measured skin temperature at the dorsocervical, supraclavicular and abdominal area, before and after 2 hours of personalized cold exposure [8] by means of infrared thermography (Fig. 2E) in a separate sample of 11 adults. Whereas the skin temperature of the abdomen decreased after cold exposure (mean change -1.4±0.9°C, P<0.001, Fig. 2F), no such change was observed in the skin temperature of the supraclavicular (+0.1±0.6°C, P=0.455) and dorsocervical area (0.0±0.6°C, P=0.924, Fig. 2F).

DISCUSSION

This study shows evidence of that the 18F-FDG uptake by SAT is different among different depots in young adults. Moreover, we found that the glucose uptake in the SAT of dorsocervical area correlated with supraclavicular BAT volume, SUVmean and SUVpeak, whereas SAT of the tricipital area did not correlate with supraclavicular BAT-related outcomes. Additionally, we found that in 23 out 133 participants presented glucose uptake in the dorsocervical area that achieved the criteria to be consider BAT[9] (dorso-PET+ group), being mainly women. In a confirmatory study, we observed that dorsocervical skin temperature was higher in comparison to the abdominal skin temperature upon a cold exposure. Moreover, we did not find differences between dorsocervical and supraclavicular skin temperature. Taken together, these findings show that the glucose uptake of the SAT is not the same in young healthy adults. Therefore, these depots should be biopsied and further study in order to elucidate their real composition.

The occurrence of 18F-FDG uptake by SAT of the dorsocervical area is consistent with the histological finding by Heaton [12]. She showed, that the SAT of the dorsocervical area was interscapular BAT (iBAT). More recently, Lidell et al. [6] studied the composition of the SAT of the dorsocervical and back area and they found that the composition of this tissue was (iBAT), (similar levels of UCP-1 and ZIC-1 compared to human classical BAT). Both studies [6,12] were carried out in corpses. Interestingly, Cereijo et al. [13] showed that individuals with the immunodeficiency virus often present lypohypertrophy in the dorsocervical area (i.e. buffalo hump). Gene expression analysis of this tissue reveals a high expression of UCP-1 and ZIC-1 [13], which suggests the presence of iBAT in these patients. Altogether, with the positive correlations between SAT of the dorsocervical area with supraclavicular BAT in the present study, make us hypothesize that maybe young adults still have active iBAT in the dorsocervical area, but this fact should be confirmed at molecular levels.

White adipose tissue (WAT) of humans, have the possibility to transdifferentiate white adipocytes into brown-like adipocytes, by a process named browning (brown-in-white) [14]. These brite/beige cells possess multilocular morphology and express the UCP-1 as a molecular hallmark of the presence of brown adipocytes [15]. The best currently known strategy to induce browning is cold exposure

[14]. In humans, the SAT of the abdomen is the most common site to analyze the potential browning effects of different kind of interventions. However, studies in humans failed to show browning of WAT after a cold-intervention [16,17]. We observed that the skin temperature of the abdomen decreased after a cold exposure, whereas the skin temperature of the dorsocervical area did not. These findings suggest that biopsies of the SAT of the abdomen might not be the best place to quantify browning in humans and findings alternative places (e.g. dorsocervical area) is of imperial need. Therefore, we showed for first time that the glucose uptake by the SAT of the dorsocervical area is higher in comparison to other SAT area. Based on the present results, we hypothesize that this high glucose uptake in this area could be iBAT, and further studies at the molecular levels are needed.

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Associations between cardiorespiratory and muscular fitness with brown adipose tissue and skeletal muscle 18F-Fluorodeoxyglucose uptake in young adults

CHAPTER 13

BACKGROUND

We introduced participants to a mild cold room (~19°C), dressed with a cooling vest with Brown adipose tissue (BAT) is present and metabolically active in humans[1–3]. BAT activation has been postulated as potential therapy to combat obesity, based on its capacity to increase the energy expenditure in mice studies[4]. Moreover, BAT seems to act as endocrine organ able to modulate the whole body metabolism[5,6]. However, the relative amount of BAT in the human body is lower in comparison to mice studies[7], and therefore its implication in the human metabolism should be different, as well as some of the strategies to activate it.

Finding feasible strategies for the activation and recruitment of BAT in humans are needed. Cold stimuli has shown to be the main activator[2,8,9], whereas other have shown that certain drugs or dietary components[10] could also activate BAT. We hypothesized that exercise might be able activate or recruit human BAT [11]. Exercise is an effective stimulus against the most of the physical[12] and mental[13] diseases, but its effect on BAT is currently uncertain [14–16]. A set of case-control studies showed that endurance-trained men[17] and women[18] had lower levels of 18F-Fluorodeoxyglucose (18F-FDG) uptake by BAT compared to sedentary controls[17]. Dinas et al. [19] showed that BAT was positively related with levels of physical activity (PA) measured by questionnaire [20]. In contrast, we found that objectively measured PA (i.e. accelerometry) was not associated with BAT volume or activity in young adults [21].

Physical fitness is a powerful marker of health and a better predictor of morbidity and mortality[22,23] than PA. The main components of physical fitness are cardiorespiratory fitness (CRF) and muscular strenght[23]. Cardiorespiratory fitness reflects the overall capacity of the cardiovascular and respiratory systems and the ability to carry out prolonged exercise. Hence, cardiorespiratory fitness has been considered as a direct measure of the physiological status of the individual. Cardiorespiratory fitness provides strong and independent prognostic information about the overall risk of illness and death in both men and women across a broad spectrum of ages[24]. Several prospective studies have shown that muscular strength is inversely associated with all-cause mortality[22]. However, whether CRF or muscular strength is associated with human BAT before is not known. We know that endurance and resistance exercises have different physiological adaptations[25], and therefore the association with BAT should be different.

Thus, we aimed to study the association of CRF and muscular strength with 18F-FDG uptake by BAT and skeletal muscle after a cold exposure in young adults.

MATERIAL AND METHODS

Research design and participants

This cross-sectional study was performed under the framework of the ACTIBATE study (activating brown adipose tissue through exercise)[26]. A total of 119 young adults participated in the present study (n=38 males). All assessments were performed in Granada (Spain) during the months of October, November and December 2015 and 2016. All participants received a comprehensive medical examination and reported to be sedentary (<20 min physical activity on <3 days/week), had a stable body weight in the last 3 months (<3 Kg change), were not exposed to cold regularly and did not smoke or take any medication. The study protocols and design were applied in accordance with the current ethical guidelines (declaration of Helsinki, last revision in 2013) and were approved by the Human Research Ethics Committee of the University of Granada (n°924) and of the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada). All participants signed an informed consent.

Procedures

Each participant came to the research center five independent days in order to perform the following tests.

Body composition measurement

We determined lean body mass (LBM) and fat mass percentage by a Dual Energy X-ray Absorptiometry (HOLOGIC, Discovery Wi). We measured body weight and height with a SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Body mass index (BMI), likewise lean mass index (LMI), were calculated as body weight divided by height square.

Cardiorespiratory fitness

Participants arrived to the research center in a fasting state 3-5 hours, they did not perform any type of exercise 48 hours before the test, and they did not consume caffeine or tea 23 hours before.

CRF was determined using a maximum treadmill exercise test (h/p cosmos, Italy) following the modified Balke protocol[26]. We measured energy expenditure before, during and after the CRF test by indirect calorimetry using a metabolic cart CPX Ultima CardioO2 (Med-graphics Corp, Minnesota, USA) using a plastic face-mask (model 7400, Hans Rudolph Inc, Kansas City, MO, USA) and equipped with a prevent™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA)[27]. The metabolic cart measure VO2 and VCO2 using a breath-by-breath technique for determining the gas exchange. VCO2 measurement is performed using a non-disperse infrared analyser, and VO2 is measured using a galvanic fuel cell[27]. A medical doctor placed the diodes for registering the electrocardiogram before, during and after the endurance exercise. Then, the medical doctor placed the face-mask and a researcher explained the test to the participant. The test had a warm-up of 1 minute at 3km/h and 2 more minutes at 4km/h. In the minute 4 the speed of the treadmill increased up to 5.3 km/h with the slope at 0%. This was the maximal speed of the treadmill. Every minute the slope of the treadmill increased 1% until the participant reached his/her maximum volume of oxygen (VO2max). The criteria for achieving VO2max were: respiratory exchange ratio ≥ 1.1, a plateau in VO2 (change of <100 ml/min in the last 3 consecutives 10 seconds stage), and a heart rate within 10 beats/min of the age-predicted maximal heart rate (209–0.73 ∗ age) [28]. We also calculated the time that participants took to achieve their VO2max (time to exhaustion in seconds). We represented the VO2max relative to LBM (ml/kg LBM/min).

Muscular strength

Muscular strength was measured by three tests: handgrip dynamometer, and 1 maximum repetition (RM) for bench and leg press.

Handgrip dynamometer: It was assessed by a handgrip dynamometer (Takei 5401 digital). Every participant were standing in a bipedal position maintaining the arm of the tested side straight down with the shoulder slightly abducted (~10° not touching the rest of the body), the elbow in 0° flexion. Each participant performed the test twice alternately with both hands with 1 min rest between attempts using the same grip span for men but individualized for women[29]. We took the average of both attempts and hands in kilograms. We represented the handgrip strength relative to LBM.

Estimation of 1-RM for upper and lower body: Upper and lower body strength were assessed by a supine bench press and a leg extension press in resistance weight machines (KEISER®). We did not perform a 1-RM measurement directly, because our participants were sedentary and they were not able to perform the technique of these exercises perfectly, which strongly affects the estimation of the 1-RM. Therefore, we used the Wathen equation, which is valid to estimate the 1 RM for bench and leg press[30] in not well trained people.

1RM=(Weight lifted per repetition (kg))/((48.8+ ⟦ 53.8e*(-0.075×number of repetitions))/100)

We normally started with the 1-RM estimation for bench press after the measurement of handgrip strength. We explained them that for a successful 1-RM estimation they should performed less than 10 repetitions with a weight that they were able to manage. They had 2 attempts for exercise for a successful estimation, if they

failed in the estimation, they needed to come in an alternative day to the research center. Following that, we encouraged to the participants to lie down in the bench and they should perform several repetitions without any weight to be familiarized with the exercise, this was the warm-up. After that, participants performed the first attempt. If they knew that she/he will be able to perform more than 10 repetitions, we stopped the exercise and the participants rest at least 5 minutes before starting the second attempt. During this rest, researchers increased the weight and after the rest, participants performed the new attempt. If the participant performed less than 10 repetitions, at his /her maximum strength capacity, we finalized the exercise and we write down the number of repetitions as well as the weight that they lifted. We performed the same process for 1RM estimation for the leg press exercise. When we obtained the data, we applied the Wathen equation in excel sheet in order to obtain the 1-RM estimation for bench and leg press. We represented the 1 RM values of the bench and leg press relative to LBM.

Positron Emission Tomography/Computed Tomography (PET/CT) quantification

An extended version of the cooling protocols that we used as well as the BAT quantification can be found elsewhere [21,31,32]. In brief, we cooled down the participants using a cooling vest (Polar Products Inc., Ohio, USA) until shivering occurred in a mild-cold room (19.5-20°C). After 48-72 hours later, participants went to the Hospital Virgen de las Nieves, where we introduced them in a room (19.5-20°C) and wearing the same cooling vest but ~4°C above their shivering threshold test during 2 hours. After the first hour we injected to the participants the 18F-FDG (~185Mbq) and we increased 1°C the water temperature in order to avoid the visually shivering. After the 2 hours of cooling we performed the PET/CT (Siemens Biograph 16 PET/CT, Siemens, Germany) quantification using 2 BED from the atlas vertebra until thoracic vertebra 6.

We quantified BAT volume and activity following the recent recommendations [33] using the Beth Israel plugin for FIJI [32-31]. We applied a fixed range of Hounsfield units (HU, −190 to −10) and an individualized standardized uptake value (SUV) threshold [1.2/(lean body mass/body mass)]. We determined BAT volume, SUVmean (as a proxy of mean 18F-FDG uptake), BAT metabolic activity and SUVpeak (the average of the 3 highest pixels of 18F-FDG uptake located in less than 1 cm) [32] as well as the 18F-FDG uptake in a reference tissue (descending aorta). We quantified the 18F-FDG uptake (SUVpeak) of several skeletal muscles included between atlas vertebrae and thoracic vertebrae 4. We drew a single ROI from 1 slice in paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, suprasipinatous, subscapular, deltoid, pectoralis major, and triceps braquis muscles from both left and right side of the body [8,34]. An average of both sides including all skeletal muscles was performed in order to obtain a single representative value of the skeletal muscle 18F-FDG uptake of the upper part of the body. Moreover, we performed different skeletal muscle groupings [34].

Statiscal analyses

The data are presented as mean and standard deviation, unless otherwise stated. We conducted simple linear regression analyses (model 1) to examine the association between CRF and muscular fitness outcomes with BAT volume and activity as well as with 18F-FDG uptake by skeletal muscles (dependent variables). We also used multiple linear regression models to test these associations adjusting by date when the PET/CT was performed (model 2), and adding sex (model 3). The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM SPSS Statistics, IBM Corporation) and the level of significance was set at <0.05.

Table 1: Characteristics of the participants

	All sample			Men			Women		
	Mean		SD	Mean		SD	Mean		SD
N (% men)	119 (31.9%)			38			81		
Age (years old)	21.9	±	2.1	22.1	±	2.2	21.8	±	2.1
Body mass index (kg/m2)	25.0	±	4.8	27.6	±	5.7	23.7	±	3.9
Lean mass index (kg/m2)	14.6	±	2.4	17.2	±	2.1	13.3	±	1.4
Fat mass percentage (%)	36.3	±	7.2	31.7	±	7.8	38.4	±	5.9
Handgrip strength (kg)	31.2	±	7.8	40.0	±	6.7	27.0	±	3.8
1-RM Leg press (kg)	200.6	±	69.4	281.3	±	50.9	162.7	±	36.9
1-RM Bench press (kg)	31.5	±	14.9	49.7	±	12.0	23.0	±	5.5
VO2max (ml/kg/min)	41.4	±	7.9	44.2	±	9.6	40.1	±	6.6
BAT volume (ml)	73.8	±	58.7	92.2	±	68.1	65.2	±	51.9
BAT SUVmean (g/ml)	3.91	±	1.91	3.64	±	1.29	4.03	±	2.13
BAT SUVpeak (g/ml)	11.79	±	8.31	11.45	±	7.50	11.95	±	8.71
Average all skeletal muscle (SUVpeak g/ml)	0.81	±	0.20	0.81	±	0.18	0.81	±	0.21
Descending aorta (SUVpeak g/ml)	1.57	±	0.33	1.66	±	0.35	1.52	±	0.32

Data are presented as mean and standard deviation. BAT: brown adipose tissue; RM: repetition maximum; SUV: standardized uptake value; VO2: maximal oxygen consumption.

RESULTS

Table 1 summarizes the descriptive characteristics of the participants.

Figure 1 shows the association between CRF (i.e. time to exhaustion and VO2max) with BAT volume and activity. Time to exhaustion was not associated with BAT volume, BAT SUVmean and BAT SUVpeak (Figure 1A: β=-0.006; R2=0.000; P=0.850, β=-0.001; R2=0.006; P=0.444 and β=-0.002; R2=0.003; P=0.592, respectively). Similarly, there was no association between VO2max and BAT volume, BAT SUVmean and BAT SUVpeak (Figure 1B: β=-0.247; R2=0.002; P=0.667, β=-0.017; R2=0.008; P=0.352 and β=-0.043; R2=0.002; P=0.598). The results persisted after controlling for date when the PET/CT was performed (model 2) and date when the PET/CT was performed and sex (model 3, Table 2).

Figure 2 shows the association of muscular strength (i.e. handgrip strength/LBM, leg press/LBM and bench press/LBM) with BAT volume and activity. Handgrip strength was not associated with BAT volume (β=83.962; R2=0.022; P=0.104, Fig 2A), whereas it was positively associated with BAT SUVmean and BAT SUVpeak: β=3.595; R2=0.039; P=0.031 and β=15.314; R2=0.037; P=0.035, Fig 2B and C). On the other hand, we did not observe a significant association between leg press with BAT volume and activity (all P≥0.218, Fig 2D, E and F), as well as between bench press and BAT (all P≥0.240, Fig 2G, H and I). The results persisted after controlling for date when the PET/CT was performed (model 2) and date when the PET/CT was performed and sex (model 3, Table 2).

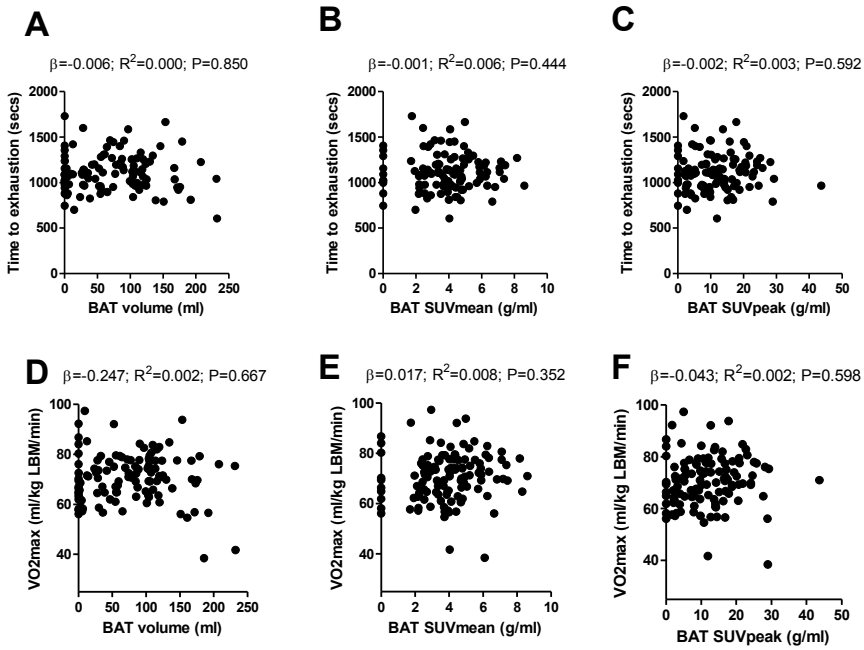

Figure 1. Associations between time to exhaustion and cardiorespiratory fitness (maximum oxygen uptake, ml/kg lean body mass /min) with brown adipose tissue (BAT) volume and activity. N=98 participants. β and P from a simple linear regression analysis. β= unstandardized coefficients; R2= explained variance.

We performed several sensitivity analyses: firstly, we repeated the analyses splitting the sample by weight status and the positive and significant associations between handgrip strength relative to LBM with BAT activity disappeared (data not shown).

Secondly, we repeated the analyses between muscular stren gth outcomes in absolute values with BAT-related outcomes and we found that all muscular strength outcomes were associated only with BAT volume in the model 1 and 2 (data not shown). We also repeated the analyses between muscular strength outcomes relative to body weight and we did not find any associations with BAT-related outcomes (data not shown).

We did not find any association between CRF with 18F-FDG uptake by skeletal muscle, WAT and reference tissue relative to LBM (table 3). Similarly, we did not find any association between muscular strength relative to LBM with 18F-FDG uptake by skeletal muscle (table 4).

DISCUSSION

This study shows that CRF is not associated with BAT volume or activity in young adults. Moreover, we observed that handgrip strength relative to LBM was positively and significant associated with BAT activity (SUVmean and SUVpeak). We observed however, no association of leg and bench press with BAT volume and activity. These observational findings should be confirmed in exercise-based intervention studies focused on improving muscular strength. This design will allow us to understand whether changed on muscular strength after the exercise intervention are associated with changes on BAT.

Two case-control studies [17,18] have addressed the relationship of the CRF measured by VO2max with human BAT. They studied the levels of BAT in a group of sedentary individuals vs. a group of well-

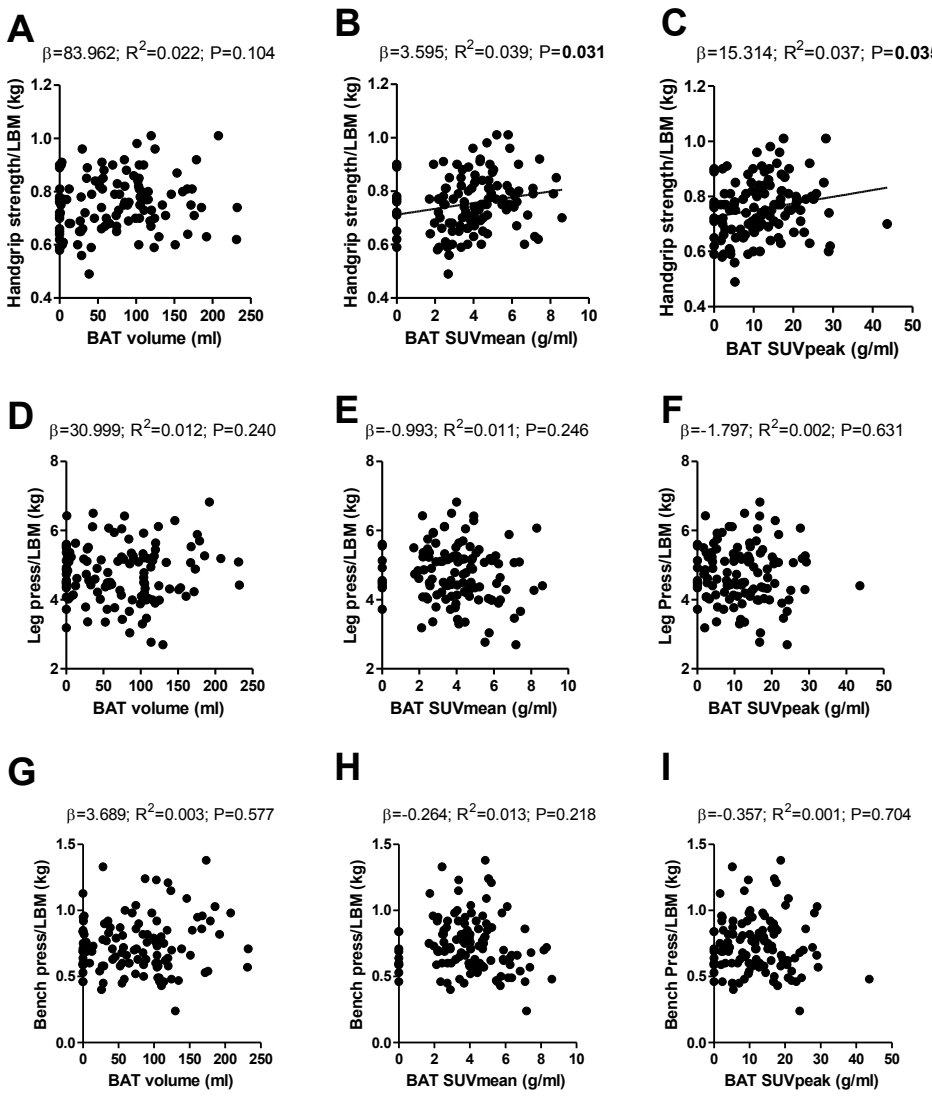

Figure 2. Associations between handgrip strength leg and bench press relative to lean body mass (LBM) with brown adipose tissue (BAT) volume and activity. N=119 participants. β and P from a simple linear regression analysis. β= unstandardized coefficients; R2= explained variance.

trained endurance men[17] and women[18] with high levels of VO2max, and showed that the well-trained group had lower BAT volume and activity. However, in the present study we did not find any association between CRF and BAT in young adults. This could be explained by the fact that our cohort is composed by sedentary people. Maybe, the long-term training level of the person could have an impact in the functionality of BAT. From a physiological point of view, the role of BAT in a well-trained person might be different from a sedentary non-exercise person although, this assumption needs to be confirmed.

Table 2. Associations of cardiorespiratory fitness and muscular strength (relative to lean body mass) with brown adipose tissue (BAT) volume and activity in young adults.

		BAT volume (ml) BM			BAT SUVmean (g/ml) BM			BAT SUVpeak (g/ml) BM		
	n	β	R2	P	β	R2	P	β	R2	P
MODEL 2										
Time to exhaustion (secs)	98	0.005	0.135	0.846	-0.001	0.144	0.101	-0.005	0.151	0.147
VO_2max (ml/kg LBM/min)	98	-0.641	0.189	0.224	0.006	0.147	0.719	-0.008	0.160	0.912
Handgrip strength/LBM (kg)	119	70.724	0.185	0.135	3.211	0.169	**0.040**	13.568	0.178	**0.045**
Leg press/LBM (kg)	119	3.257	0.172	0.591	-0.277	0.153	0.166	-0.414	0.151	0.633
Bench press/LBM (kg)	119	21.807	0.175	0.368	-1.275	0.157	0.110	-3.057	0.155	0.379
MODEL 3										
Time to exhaustion (secs)	98	-0.004	0.145	0.884	-0.001	0.168	0.285	-0.004	0.159	0.273
VO_2max (ml/kg LBM/min)	98	-0.541	0.219	0.299	0.004	0.165	0.828	-0.014	0.166	0.851
Handgrip strength/LBM (kg)	119	67.768	0.220	0.145	3.277	0.186	**0.035**	13.701	0.182	**0.043**
Leg press/LBM (kg)	119	-3.875	0.207	0.565	-0.184	0.158	0.415	-0.205	0.152	0.836
Bench press/LBM (kg)	119	-28.079	0.210	0.389	-0.928	0.159	0.396	-2.936	0.155	0.538

Model 2: adjusted by date when positron emission tomography/computed tomography (PET/CT) was performed. Model 3: adjusted by date of PET/CT and sex. β and P from a simple linear regression analysis. β= unstandardized coefficients; BM= Body mass; LBM: lean body mass; SUV= Standardized uptake value; R2= explained variance; VO2= volume of oxygen.

Several studies have analysed the effect of endurance training on BAT[11,35]. However, less attention has been paid to resistance training. Endurance and resistance exercise have different physiological adaptations in humans[25]. For instance, resistance exercise is the most natural anabolic stimulus for skeletal muscles and has shown to increase muscle mass[36] and energy expenditure in humans[37]. The fact that the results of the present study show that handgrip strength is positively associated with BAT open the debate that further and alternative studies studying the role of resistance training in human BAT are needed. For instance, studies focuses on the type of exercise (endurance vs. resistance), intensity (moderate vs. vigorous), or the status of the person (untrained vs. trained individuals) are of interest[11]. Interestingly, we did not find the same positive association between the other study parameters of muscular strength (1-RM estimated for bench and leg press) with 18F-FDG uptake by BAT, despite the fact that the correlation between Handgrip strength and 1-RM estimations for bench and leg press were above 0.4 r of Pearson. Several studies have shown that handgrip strength is one of the best muscular strength markers and that it predicts the risk of mortality[22,38–40]. The differences observed in the association of handgrip strength and bench and leg press with BAT could be explained because the technique needed for performing a handgrip strength repetition is a movement that all people normally do in their normal life (shaking the hand, carrying the bags of the supermarket to their houses, grapping things, etc.). However, bench and leg press are specific exercise that people can do once they know the technique. In our cohort, all people were sedentary and for the vast majority these evaluations were the first experience with the bench and leg press and this could be explaining our results differences. Nevertheless, further studies are needed because as we mentioned above handgrip strength correlated moderate with bench and leg press.

On the other hand, white adipose tissue (WAT) has the possibility to be transdifferentiate to brown-like cells by a process commonly named as browning[41]. These cells are known as BRITE (brown-in-white). Animals studies have shown that endurance exercise induce browning of the WAT more than a possible activation of the classical BAT[35]. In humans, whether exercise is able to induce browning seems controversial. Vosselman et al.[17] did not find differences in browning markers of the subcutaneous WAT of the abdomen between well-trained people vs. control group. In contrast Dinas et al.[42] observed that those people who reported higher levels of PA had higher browning markers in the subcutaneous WAT of the abdomen. Therefore, further studies focus on the relationship between physical fitness and browning are of interest.

This study presents several limitations. This is a cross-sectional, observational study; therefore, we cannot infer any cause-effect relationship. The correlation coefficient of the

positive associations between handgrip strength with BAT activity are weak, although seem constants upon the different statistically models. We have used 18F-FDG, a glucose analogue and we know that BAT consumes more fatty acids rather than glucose[43]. Therefore, replicating these findings using others tracers is of interest[44], as well as in other population.

CONCLUSIONS

CRF was not associated with BAT-related outcomes, whereas handgrip strength relative to LBM strength was positively associated with 18F-FDG uptake by BAT activity (SUVmean and SUVpeak). We did not find any relationship between CRF and muscular fitness with other tissues. Intervention studies are need to elucidate the role of the muscular strength as possible BAT/browning activator in humans.

Table 3. Associations between cardiorespiratory fitness outcomes with skeletal muscles, dorsocervical and reference glucose uptake outcomes in 98 young healthy adults.

	Deep skeletal muscles (SUVpeak) BM			Superficial skeletal muscles (SUVpeak) BM			Cold sensitive skeletal muscles (SUVpeak) BM			Average skeletal muscles (SUVpeak) BM			Dorsocervical WAT (SUVpeak) BM			Descending aorta (SUVpeak) BM		
	β	R2	P	β	R2	P	β	R2	P	β	R2	P	β	R2	P	β	R2	P
MODEL 1																		
Time to exhaustion (secs)	-0.001	0.038	0.055	-0.001	0.113	0.001	-0.001	0.026	0.110	0.000	0.032	0.080	0.000	0.003	0.571	0.000	0.000	0.833
VO_2max (ml/kg LBM/min)	0.002	0.001	0.694	-0.003	0.008	0.353	0.004	0.004	0.477	0.000	0.001	0.792	0.000	0.000	0.815	-0.002	0.015	0.190
MODEL 2																		
Time to exhaustion (secs)	-0.001	0.056	0.041	-0.001	0.113	0.001	-0.001	0.035	0.093	0.000	0.036	0.093	0.000	0.004	0.566	0.000	0.007	0.776
VO_2max (ml/kg LBM/min)	0.001	0.034	0.916	-0.003	0.009	0.335	0.003	0.019	0.605	0.000	0.016	0.948	0.000	0.003	0.881	-0.002	0.015	0.208
MODEL 3																		
Time to exhaustion (secs)	-0.001	0.061	0.076	-0.001	0.120	**0.003**	0.000	0.047	0.178	0.000	0.068	0.245	0.000	0.045	0.918	0.000	0.237	0.273
VO_2max (ml/kg LBM/min)	0.000	0.055	0.968	-0.003	0.039	0.253	0.002	0.044	0.718	0.000	0.049	0.805	0.000	0.029	0.788	-0.002	0.191	0.355

Model 1: Unadjusted. Model 2: adjusted by date when positron emission tomography/computed tomography (PET/CT) was performed. Model 3: adjusted by date of PET/CT and sex. β and P from a simple linear regression analysis. β= unstandardized coefficients; LBM= lean body mass; R2= explained variance; SUV= Standardized uptake value; LBM: lean body mass; VO2= volume of oxygen.

Table 4. Associations of muscular strength (relative to lean body mass) with skeletal muscles, dorsocervical withe adipose tissue and descending aorta glucose uptake outcomes in 119 young healthy adults.

	Deep skeletal muscles (SUVpeak)BM			Superficial skeletal muscles (SUVpeak)BM			Cold sensitivity skeletal muscles (SUVpeak)BM			Average skeletal muscles (SUVpeak)BM			Dorsocervical (WAT) (SUVpeak)BM			Descending aorta (SUVpeak)BM		
	β	R2	P	β	R2	P	β	R2	P	β	R2	P	β	R2	P	β	R2	P
MODEL 1																		
Handgrip strength/LBM (kg)	0.199	0.005	0.459	-0.119	0.007	0.350	0.293	0.009	0.293	0.028	0.000	0.871	0.008	0.000	0.972	-0.254	0.006	0.386
Leg press/LBM (kg)	0.008	0.000	0.821	0.006	0.001	0.706	-0.011	0.001	0.758	0.000	0.000	0.989	0.003	0.000	0.901	0.036	0.008	0.331
Bench press/LBM (kg)	-0.020	0.000	0.884	-0.100	0.020	0.122	-0.022	0.000	0.877	-0.083	0.007	0.353	-0.093	0.006	0.395	0.075	0.002	0.614
MODEL 2																		
Handgrip strength/LBM (kg)	0.170	0.035	0.525	-0.118	0.008	0.356	0.273	0.022	0.327	0.013	0.019	0.940	0.003	0.003	0.990	-0.252	0.007	0.393
Leg press/LBM (kg)	0.007	0.032	0.842	0.006	0.001	0.706	-0.012	0.015	0.743	0.000	0.019	0.993	0.004	0.003	0.898	0.036	0.008	0.331
Bench press/LBM (kg)	-0.041	0.033	0.762	-0.100	0.020	0.126	-0.037	0.015	0.796	-0.094	0.028	0.291	-0.098	0.010	0.371	0.078	0.003	0.605
MODEL 3																		
Handgrip strength/LBM (kg)	0.169	0.035	0.527	-0.117	0.008	0.361	0.277	0.025	0.321	0.015	0.020	0.933	-0.001	0.008	0.995	-0.269	0.044	0.354
Leg press/LBM (kg)	0.008	0.032	0.841	0.010	0.003	0.594	-0.004	0.016	0.920	0.004	0.020	0.884	-0.008	0.009	0.797	0.000	0.037	0.995
Bench press/LBM (kg)	-0.085	0.034	0.649	-0.169	0.032	0.058	0.024	0.017	0.902	-0.142	0.031	0.244	-0.299	0.043	0.047	-0.251	0.050	0.215

Model 1: Unadjusted. Model 2: adjusted by date when positron emission tomography/computed tomography (PET/CT) was performed. Model 3: adjusted by date of PET/CT and sex. β and P from a simple linear regression analysis. β= unstandardized coefficients; BM=body mass; LBM=lean body mass; R2= explained variance

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Distribution of brown adipose tissue, the thermoregulatory system and physical activity and fitness in metabolically healthy but overweight-obese adults: a case-control study

CHAPTER 14

BACKGROUND

Obesity has become pandemic, and estimates indicate that the prevalence of obese individuals will reach 18% by 2025 [1]. Obesity is associated with a number of cardio-metabolic alterations, however, there is a group of obese individuals characterized by a lower risk of obesity-related cardio-metabolic complications, the so called metabolic healthy but obese (MHO)[2,3][4]. MHO individuals have a healthy metabolic profile, i.e. do not have dyslipidemia, hyperglycemia, hypertension or type 2 diabetes[2,3][4], whereas their counterparts that present any of these conditions are known as metabolically unhealthy obese (MUO)[4].

Brown adipose tissue (BAT)[5–7] is a thermogenic tissue highly involved in the thermoregulatory system of murine models, generating heat upon a cold exposure and helping their bodies to preserve the core body temperature [8]. Moreover, BAT seems to be involved in the control of energy homeostasis [9], albeit the main function of this tissue in humans remains unknown[10]. In addition, the relationship between BAT and obesity seems to be controversial[5,11]. Recently U Din et al.[12] found that the average of BAT radiodensity (an indirect measurement of the fat content of the tissue) was negatively related with obesity. Whether the distribution of BAT along the different ranges of radiodensity differs in MHO and MUO is however not known. The role of BAT in the human thermoregulatory system has not been deeply studied, and it is biologically plausible that its role in MHO differs in comparison to MUO. Moreover, understanding the relation between the thermoregulation system and energy homeostasis has a potential relevance for the treatment of obesity or type 2 diabetes[13].

There is mounting evidence indicating that high levels of physical activity (PA) and fitness have strong health benefits [14,15]. A recent meta-analysis shows that MHO individuals seems to have higher levels of PA and spend less time in sedentary behaviour in comparison to MUO counterparts[16]. In addition, MHO have higher levels of cardiorespiratory fitness than MUO, whereas no differences were observed on muscular strength. Of note is that most of the available studies included were in older adults (>45 years old)[16], and whether the same findings applied to younger adults remains to be elucidated.

The aim of the present study was to determine differences in the distribution of BAT and in the thermoregulatory responses, levels of PA and fitness in metabolically healthy overweight-obese (MHOO) vs. metabolically unhealthy overweight-obese (MUOO) young adults.

MATERIAL AND METHODS

A total of 60 young adults were included in the present study (n=26; 43.3% men). Participants were selected among the cohort of the ACTIBATE study (Clinical Trials.gov ID: NCT02365129)[17]. All participants were non-smokers, were not enrolled in a weight loss program, they reported to have a sedentary lifestyle, had no acute or chronic illness and reported not to be regularly exposed to cold. We classified participants as MHOO or MUOO [18]. MHOO was defined as having a BMI ≥ 25 kg/m2 and no presenting any of the following criteria: a) Serum triglyceride concentration ≥ 150mg/dL; b) High-density lipoprotein cholesterol (HDL) concentration <40 mg/dL for men and 50 mg/dL for women; c) Systolic blood pressure (SBP) ≥ 130 mmHg or diastolic blood pressure (DBP) ≥ 85 mmHg; d) Serum glycaemia >100 mg/dL. MUOO was defined as having a BMI ≥ 25 kg/m2 and presenting at least one of the above-mentioned risk factors. The study was conducted in Granada (Spain) between October and November of two consecutive years (2015 and 2016). The study protocol and informed consent were approved by the the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada) and the Human Research Ethics Committee of both the University of Granada (n° 924). A written informed consent was obtained from every participant following the Declaration of Helsinki (revision of 2013). All the measurements were performed in a total of 6 visits: i) clinical measurements (1 visit); ii) blood sample collection (1 visit); iii)

personalized cooling protocols and thermal perception (2 visits); Physical fitness (2 visits). iButtons for measuring wrist skin temperature (WT) and the personal level of environmental temperature (Personal-ET) were given in the first visit, as well as the accelerometers. These devices were collected the visit of the BAT quantification.

Clinical measurements

Participants filled up three visual analogue scales (VAS) regarding their thermal perception before their enrolment[17]. In these VAS scales 0 was "not cold at all" and 100 mm was "so much cold". One of these questions was "How much cold did you feel on your body during the last month?" we repeated the same question regarding feet and hands[19]. After that, all participants went through a deep medical examination. We measured body weight and height using a SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Moreover, blood pressure was measured following standardized protocols using an automatic sphygmomanometer Omrom M2 (Omron Healthcare, Kyoto, Japan) with appropriate cuff at 3 different consecutive times. The 3 readings were averages and we determined SBP and DBP. We measured body composition by a DEXA scan (Discovery Wi, Hologic, Inc., Bedford, MA, USA). We calculated body mass index (BMI) as well as lean mass index (LMI) (kg/m2). We also quantified the body surface area (BSA) as DuBois suggested[20].

Blood sample collection

Participants came to the research center in fasting state of 12 hours in the morning where we took 50 ml of blood. Serum samples were immediately centrifuged and store in -80°C until analyses. Glucose, insulin, HDL, total cholesterol (TC) and triglycerides (TG) were measured using specific reagents by Beckman Coulter Diagnostics. All the samples were processed in an analyzer (Beckman Coulter AU5832). Insulin was analyzed by UniCel Dxl 600 (Beckam Coulter). Homeostasis model assessment index (HOMA) and low density lipoprotein cholesterol (LDL) were calculated. All samples were evaluated in the Hospital Virgen de las Nieves, Granada, Spain.

Personalized cooling protocols and thermal perception

The personalized cooling protocol used in this study has been described elsewhere [21]. Briefly, we introduced the participants in a mild-cold room (around 19.5°C) with a water perfused cooling vest (Polar Products Inc., Ohio, USA). We decreased the water temperature gradually (0.6°C every 10 minutes) until shivering occurred. We determined the shivering threshold visually and self-reported by the participants. In this study day, we included thermal perception measurement of the participants at the beginning and to the end of test. We used a continuous 7-points thermal sensation interval scale (American Society of heating, refrigerating, and air conditioning engineers, ASHRAE)[22,23], were -3 was cold, 0 was neutral and 3 was hot. Participants also reported the subjective perception of shivering in a numeric rate scale (NRS) where 0 refers to "I am not shivering" and 10 refers to "I am shivering a lot". This perception was also collected before starting and finishing the shivering threshold determination.

Forty eight-seventy two hours later, participants went to the hospital, where we introduced them in a similar room and we exposed them to 2 hours of personalized cold exposure. The water temperature was stablished to 3.8°C above their shivering threshold temperature. After one hour of cold exposure, we injected 185MBq of 18F-fluorodeoxyglucose (18F-FDG) and we increased 1°C the water temperature and we kept the participants for another hour exposed to the cold. After the two hours of cold exposure, we introduced the participants in the positron emission tomography combined with computed tomography scan (PET/CT; Siemens Biograph 16 PET/CT, Siemens Germany). Two bed positions were scanned from approximately atlas vertebrae to thoracic vertebrae 6.

PET/CT analyses

The PET/CT images were analyzed using the Beth Israel plugin for FIJI [7] software (by BMT) under the supervision of a nuclear medicine physician (JMLL) [24]. The regions of interest (ROIs) were semi-automatically outlined from atlas vertebrae (Cervical 1) to thoracic vertebrae 4 using a 3D-Axial technique [25]. We calculated the standardized uptake value (SUV) as 18F-FDG uptake (kBq/mL)/(injected dose [kBq]/patient weight [g])]. We defined BAT volume, SUVmean, and SUVpeak following BARCIST 1.0 criteria [(SUV IND= (1.2/(lean body mass/body mass)). The Beth Israel plugin for FIJI software has the option to export a csv file with the single value of Hounsfield units (HU) and SUV for every active pixel inside the ROIs. We quantified BAT volume and activity in every single range of HU (from -10 to -11, from -11 to -12, and so on until -300). Once we identified all the pixels located in the range of HU, we applied the SUV IND threshold in the range from -10 to -300 HU [26] for BAT quantification. In order to quantify adipose tissue radiodensity we did not apply any SUV threshold with the idea of including both WAT and BAT.

Skin temperature

We measured skin temperature with 17 iButtons [27] (DS 1922 L, Thermochron; resolution: 0.0625°C; Maxim, Dallas, USA). We attached the iButtons to the skin with adhesive tape at different body sites [27,28]. We recorded the skin temperature at 1-min intervals. The mean, proximal, distal and supraclavicular skin temperature were estimated as we proposed recently[28]. Moreover, a peripheral gradient was calculated as a proxy of peripheral vasoconstriction (forearm-fingertip) [27]. All data registered by the devices and equations were analysed by the Temperatus® software. We attached the iButtons before starting the test, after the standardized individualized meal intake, we recorded the thermic effect of food during 3 hours [29] as well as the effect of cooling protocol.

During the previous measurements, we collected from the Spanish National Meteorological Agency the average temperature of Granada for every day, and then we performed an average in order to calculate the outdoor ambient temperature. Participants wore 24 hours during 7 days an iButton placed on the ventral wrist (WT) and an iButton placed in plastic devices in order to quantity the Personal-ET. All data were analysed by Temperatus® software and an average of 7 days were performed.

PA and sedentary time

A detailed description of the PA assessment has been described elsewhere[30]. In brief, 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) [31]. The accelerometers were initialized to store raw accelerations at a sampling frequency of 100 Hz [32]. 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 GGIR package estimated the time spent in sedentary behavior and in different PA intensities [light (LPA), moderate (MPA), vigorous (VPA), and moderate-vigorous (MVPA)] using age-specific cut-points for Euclidean norm minus one (ENMO) [33,34]. We also calculated the time spent in moderate-vigorous PA in bouts of ≥ 10 minutes (MVPA10min) 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.

Physical fitness: muscle strength and cardiorespiratory fitness

Muscle strength: It was assessed by a handgrip dynamometer (Takei 5401 digital)

as described elsewhere[35]. The participants were standing in a bipedal position maintaining the arm being tested straight down with the shoulder slightly abducted (~10° not touching the rest of the body), and the elbow in 0° flexion. The participants performed the test twice alternately with both hands with 1 min rest between attempts.

Cardiorespiratory fitness: It was determined using a maximum treadmill walking test (h/p cosmos, Italy) following the modified Balke protocol[17]. We measured energy expenditure by indirect calorimetry using a metabolic cart Ultima CardioO2 (Med-graphics Corp, Minnesota, USA), using a plastic face-mask without external ventilation[36]. The metabolic cart measures VO2 and VCO2 using a breath-by-breath technique for determining the gas exchange. VCO2 measurement is performed using a non-disperse infrared analyser, and VO2 is measured using a galvanic fuel cell[36]. Participants arrived to the research center in a fasting state 3-5 hours, no exercise 48 hours before the test, and without consuming caffeine or tea for the last 24 hrs. A medical doctor placed the diodes for registering the electrocardiogram during the test. The test started with a warm-up of 1 minute at 3km/h and 2 more minutes at 4km/h. In the 4th minute the speed increased up to 5.3 km/h with the slope at 0°. This was the maximal speed of the treadmill. Every minute the slope of the treadmill increased 1° until the participant reached his/her maximum volume of oxygen (VO2max). The criteria for achieving maximum oxygen consumption (VO2max) was: respiratory exchange ratio ≥1.1, a plateau in VO2 (change of <100 ml/min in the last three consecutive 10 s stage), and a heart rate within 10 beats/min of the age-predicted maximal heart rate.

Statistical Analysis

We compared sex frequency between MHOO and MUOO using Chi-square test. Since there was a higher proportion of women in the MHOO than in the MUOO group (n=28, 71.8%; n=6, 28.6%, respectively, P=0.002), all the subsequent analyses were adjusted by sex. We conducted one-way analysis of covariance (adjusting by sex) to study the differences between MHOO vs. MUOO in time to shivering, the water temperature during the shivering threshold test, thermal perception scales, 18F-FDG uptake in the subcutaneous white adipose tissue of the dorsocervical and tricipital areas, PA levels and fitness levels. To study the interaction effect of the phenotype (MHOO vs. MUOO) on adipose and BAT radio-density, we conducted a two-way ANOVA with repeated measurements. We introduced the amount of BAT calculated every 10 HU (for instance, from 0 to -10, and so on; 30 levels) as within-subject factor and the phenotype as between-subject factors. P for main effect and interaction (Time*Group effect) was used the correction of Huynh-Feldt. We repeated the same analyses for skin temperature parameters. All analyses were performed using the IBM SPSS Statistics for Windows version 22.0 (Armonk, NY: IBM Corp), and the level of significance was set to P<0.05.

RESULTS

Table 1 shows the characteristics of the participants.

Figure 1A shows that the MHOO participants were exposed to a similar outdoor ambient temperature, as well as they were exposed to the same Personal-ET in comparison to the MUOO individuals (mean difference MHOO minus MUOO: -3.7°C, 95% confident interval (CI): -7.3°C / -0.06 °C, P=0.089; and -2.1°C; -5.1°C/1.1°C; P=0.07, respectively). There were no differences on WT between MHOO and MUOO (-0.77°C, -1.4°C / -0.9°C; P=0.195). Despite the fact that both phenotypes were exposed to the same temperatures, MHOO perceived colder the body, feet and hands

Table 1. Descriptive characteristics of metabolically healthy but overweight-obese (MHOO) and metabolically unhealthy but overweight-obese (MUOO) participants.

	MHOO			MUOO			P
	n (% men)			n (% men)			
Age (years-old)	39 (28.2%)	22.1	0.4	21 (71.4%)	23.1	0.5	0.127
BMI (Kg/m2)	39	28.9	0.5	21	30.3	0.7	0.270
LMI (Kg/m2)	38 (26.3%)	16.3	0.2	19 (73.7)	16.2	0.3	0.913
VAT volume (ml)	38	469.1	23.6	19	538.1	34.7	0.122
Fat mass (%)	38	39.9	0.8	19	41.0	1.2	0.452
BSA (m2)	39 (28.2%)	2.0	0.0	21 (71.4%)	2.0	0.0	0.728
Glucose (mg/dl)	39	87.1	1.1	21	94.7	1.6	**<0.001**
Insulin (µUI/ml)	39	9.5	1.1	21	15.2	1.5	**0.010**
HOMA index	39	2.1	0.3	21	3.7	0.4	**0.008**
HDL (mg/dl)	39	49.9	1.6	21	46.6	2.3	0.271
LDL (mg/dl)	39	92.7	4.7	21	112.9	6.5	**0.018**
TC (mg/dl)	39	156.2	5.9	21	186.2	8.3	**0.007**
TG (mg/dl)	39	65.2	9.9	21	162.1	13.9	**<0.001**
SBP (mmHG)	38 (26.3%)	120.5	1.9	21	129.5	2.5	**0.008**
DBP (mmHG)	38	69.2	1.2	21	75.1	1.7	**0.013**

Data are means and standard mean error. BMI: Body mass index; BSA: body surface area; LMI: Lean mass index; HOMA: Homeostasis model assessment; HDL: High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; TC: Total cholesterol; TG: Triglycerides; SBP: systolic blood pressure; DBP: diastolic blood pressure; VAT: visceral adipose tissue. Data are presented as mean and standard error. P from analyses of covariance adjusting by sex.

the month before the enrolment in comparison to MUOO (Figure 1B: 2.3 mm, -12.5 mm / 16.9 mm, P=0.002; 7.8 mm, -11.2 mm / 26.7 mm, P=0.002; and 5.7 mm, -11.5 mm / 22.9 mm, P=0.010, respectively). The mean different in the time to shivering between MHOO and MUOO was -22.9 min (MHOO minus MUOO 95%CI: -47.9 min / 2.1 min; P=0.060, Figure 2A). The water temperature during the shivering threshold test was similar between groups (1.3°C; 0.1°C/ 2.5°C; P=0.073, Figure 2B), as well as the water temperature before BAT quantification (data not shown). In warm conditions, we found some differences between groups in their thermal perception (Fig. 3A and C; all P≤0.023). However, at the end of shivering threshold test, MHOO perceived colder the stimuli in comparison to the MUOO (Fig. 3B). MHOO felt colder their hands (-0.34 points; -0.75 points / 0.06 points; P=0.051), feet (-0.05 points; -0.36 points / 0.26 points; P=0.002), clavicular (-0.35 points; -0.83 points/ 0.12 points; P=0.040) and whole body (-0.22 points; -0.64 points/ 0.20 points; P=0.020) in comparison to MUOO, after cold exposure (Fig. 3B). Moreover, the MHOO perceived significant higher levels of shivering in comparison to MUOO at the end of the test (Fig. 3D; 1.1 points; -0.2 points / 2.3 points; P=0.047). Figure 4A shows that the distribution of the adipose tissue radiodensity is similar in MHOO and MUOO (P=0.375) in the range from -10 to -300 HU. However, when we applied a SUV individualized threshold, the MHOO group had a significantly higher distribution of BAT volume in the range from -10 to -100 HU (P=0.036; Fig. 2B). In addition, BAT activity (SUVmean) was higher in all ranges of BAT radiodensity (P=0.033; Fig. 4C). Figure 5A shows that the 18F-FDG uptake by the subcutaneous white adipose tissue (WAT) in the tricipital area was similar in MHOO and MUOO (mean difference: 0.03g/ml; 95%Confident interval: -0.01g/ml – 0.07 g/ml;

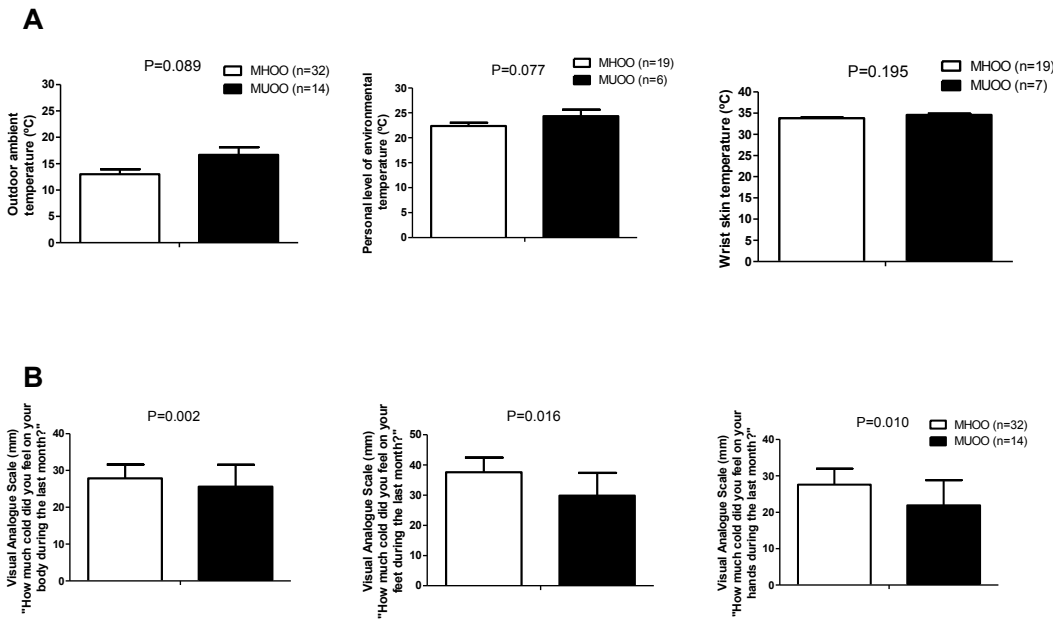

Figure 1. A. ambient temperatures and wrist skin temperature during 1 week before brown adipose tissue assessment in metabolically healthy but overweight-obese (MHOO) and metabolically unhealthy but overweight-obese (MUOO). B. Visual analogue scale in mm that participants filled up before their enrollment. They were asked about how much cold they felt in their body, feet and hands during the last month. zero means not cold at all, whereas 100 so much cold. Data are adjusted means and standard error. P values from analysis of covariance adjusting by sex.

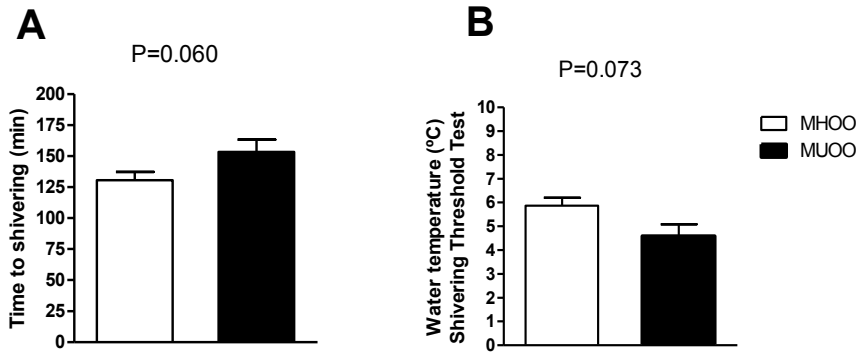

Figure 2. A. Time to shivering and water temperature at the end of the shivering threshold test in metabolically healthy but overweight-obese (MHOO, n=37) and in metabolically unhealthy but overweight-obese (MUOO, n=19) groups. Data are adjusted means and standard error. P values from analysis of covariance adjusting by sex.

P=0.154). However, the 18F-FDG uptake by the subcutaneous WAT of the dorsocervical area, was significant higher in the MHOO group (Fig 5B: 0.109 g/ml; 0.01 g/ml - 0.20 g/ml; P=0.020). There were no differences in the 18F-FDG uptake by the descending aorta (reference tissue) (MHOO=1.70±0.05 g/ml vs. MUOO=1.74±0.07 g/ml; P=0.675). Figure 6A shows the skin temperature kinetics upon a meal intake. We did not observe any increase of skin temperature after the intake of the meal in mean, proximal, distal and

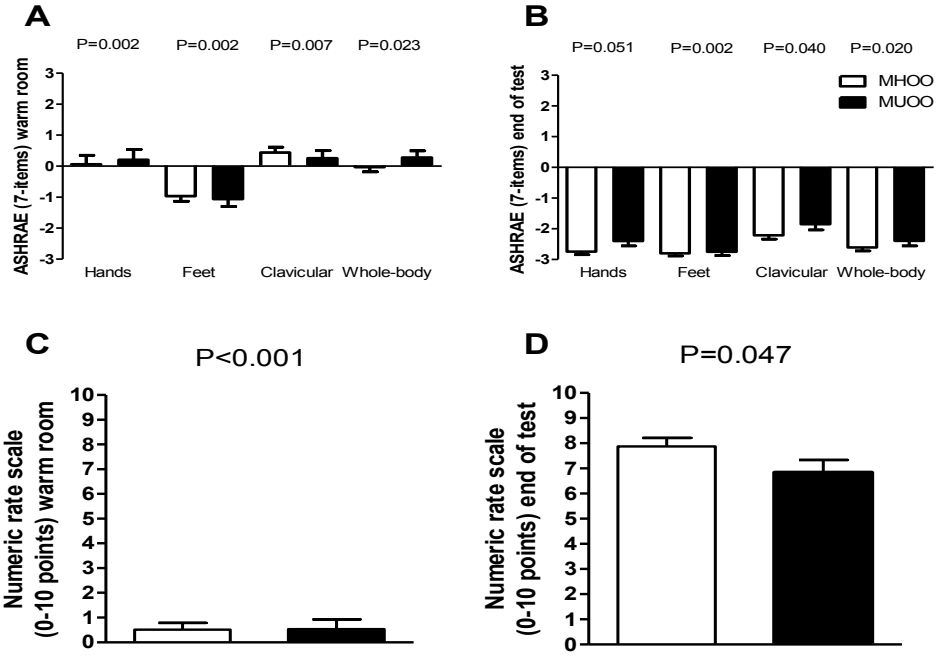

Figure 3. Thermal perception before (A and C) and after the shivering threshold test (B and D). American Society of heating, refrigerating and air conditioning engineers (ASHRAE) scales of 7 points: −3 = cold, 0 = neutral, and 3 = hot. C and D numeric rate scale (NRS): 0 = "I not shivering" and 10 = "I am shivering a lot" in both warm and at the end of the test in metabolically healthy but overweight-obese (MHOO, n=37) and in metabolically unhealthy but overweight-obese (MUOO, n=19) groups. Data are adjusted means and standard error. P values from analysis of covariance adjusting by sex.

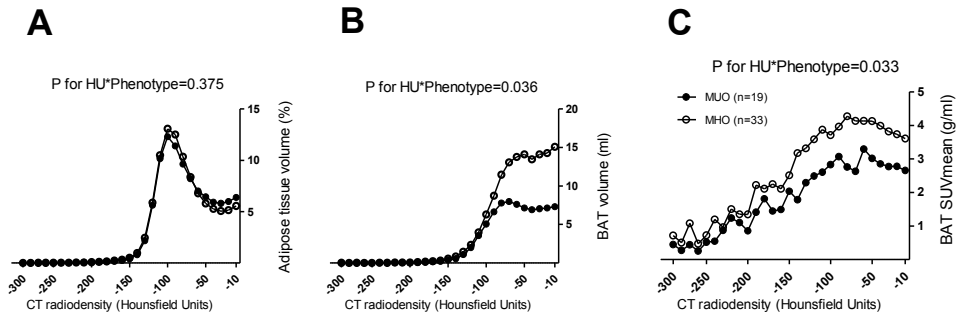

Figure 4. Brown adipose tissue radiodensity in metabolically healthy but overweight or overweight-obese (MHOO) (n=33) and metabolically unhealthy but overweight-obese (MUOO) (n=19) participants. A. differences in distribution of radiodensity in fat volume (without apply any criteria for BAT detection). B. differences in distribution of radiodensity of brown adipose tissue (BAT) volume. C. differences in distribution of radiodensity of BAT activity (SUVmean). Analyses of variance of repeated measurement were performed. All analyses were adjusted by sex. Error bars represent 95% confident interval. P for main effect and interaction (Time*Group effect) correction of Huynh-Feldt. SUV: standardized uptake value.

supraclavicular skin temperature (all P≥0.225), as well as any differences between phenotypes (all P≥ 0.107). However, there was a peripheral vasoconstriction after the meal intake (P=0.031) in both phenotypes (P= 0.561). Figure 6B shows the skin temperature kinetics upon the determination of shivering threshold test. We observed that all skin temperature parameters decreased upon cold exposure (all P≤0.013). Nevertheless, supraclavicular skin temperature was the only parameter that were different between phenotypes being higher in the MHOO group (P<0.001). In this cohort supraclavicular skin

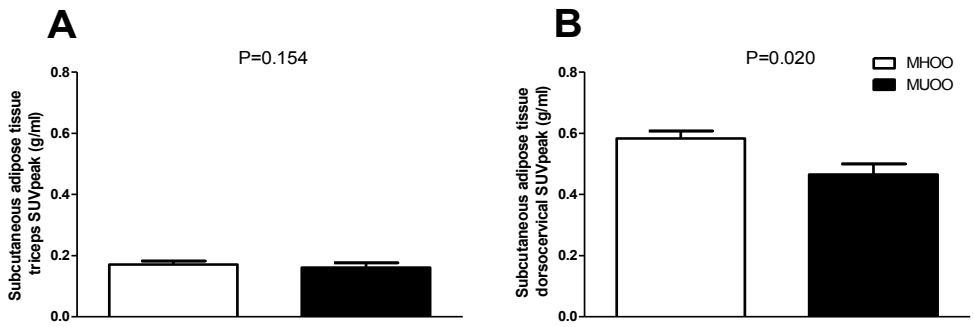

Figure 5. Subcutaneous white adipose tissue (WAT) in metabolically healthy but overweight-obese (MHOO) (n=34) and metabolically unhealthy but overweight-obese (MUOO) (n=19) participants. A. presents the differences in 18F-Fluorodeoxyglucose in the WAT of the triceps area, whereas B. presents the differences in the dorsocervical area. Dara are adjusted means and standard error. P values for analysis of covariance adjusting by sex. SUV: Standardized uptake value.

temperature at the end of the cold exposure positively correlated with BAT activity only at the end of the cold exposure (SUVmean and SUVpeak; β=0.75; R2=0.17; P=0.014 and β=3.29; R2=0.11; P=0.051, see Fig. 7A and 7B). Figure 8 shows that MHOO and MUOO groups wore the accelerometers the same hours per day. There were no MHOO vs. MUOO differences in any of the PA study dimensions (Fig. 8, all P≥0.505). Levels of handgrip strength (Fig. 9A and B, all P≥0.079) as well as CRF (Fig. 9C and D, all P≥0.145) were similar between MHOO and MUOO.

DISCUSSION

The present study shows that MHOO had higher levels of BAT volume and activity in all ranges of HU in comparison to MUOO individuals. We also observed that the levels of 18F-FDG uptake by the dorsocervical area were significantly higher in MHOO, whereas there was no difference in the levels of 18F-FDG uptake by the tricipital area, as well as by the reference tissue. Both groups were exposed to same ambient temperature before BAT quantification, and they spent similar time to shiver upon a cold-exposure. However, MHOO individuals perceived colder their body, feet and hands the month before their enrolment, as well as they perceived colder different parts of the body after the shivering threshold test. Both physical activity and fitness levels were similar between groups.

BAT and the thermoregulatory system in MHOO vs. MUOO

Cold has been postulated as a tool to combat type 2 diabetes[37] and cardiovascular disease[38], although there are controversial findings about its effect on human's health[38]. Cold exposure is not comfortable for humans. The thermal discomfort widely varies between individuals[39] and could be an important part of the thermoregulatory system which help to keep constant the core body temperature[8]. In our study, we observed that the MHOO group spent on average 22.9 min less in comparison to the MUOO to report shivering, despite the fact they had higher BAT volume and activity. At the same time, MHOO perceived colder the parts of their bodies after this cooling protocol, as well as they perceived their body colder one month before the enrolment.

These findings might indicate that the MHOO phenotype has a more sensitive thermoregulatory system. MHOO might be more sensitive to the perception of cold, which is mainly perceived by the proportion of transient receptor potential cation channel subfamily M member 8 (TRPM8). This thermal receptor is mainly hosted in the skin and the amount of them could varied between individuals[8,13]. Moreover,

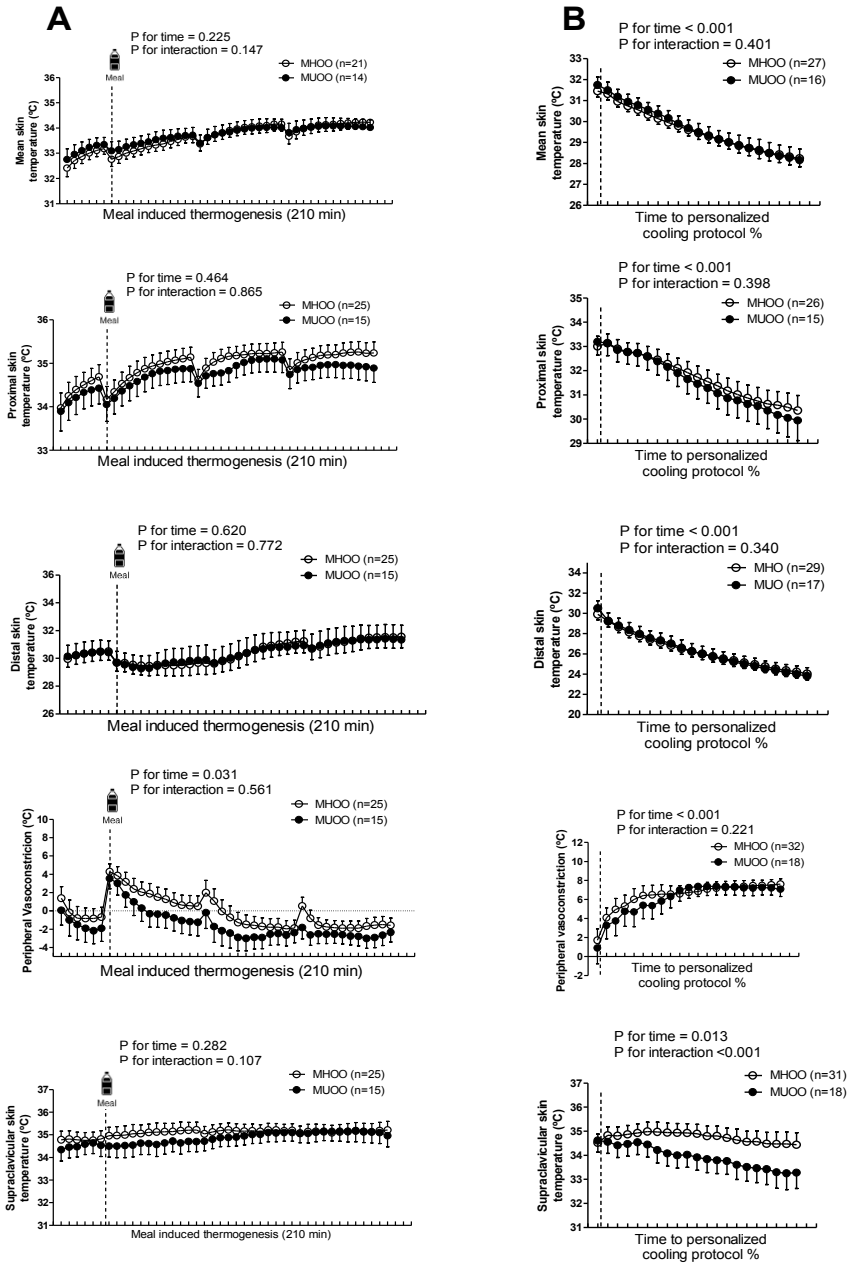

Figure 6. Kinetics of mean, proximal, distal, peripheral gradients and supraclavicular skin temperature during a meal-induced thermogenesis test (A) and time to shivering test (B) in metabolic healthy but overweight-obese (MHOO) and in metabolic unhealthy but overweight-obese (MUOO) participants. Dash line means the moment that finished the thermoneutral or baseline period. Analyses of variance of repeated measurement were performed. All analyses were adjusted by sex. Error bars represent 95% confident interval. P for main effect and interaction (Time*Group effect) correction of Huynh-Feldt

hypothalamus controls several functions in the human metabolism including thermal physiology[8,40,41]. Studies in mice show that obesity could induce a hypothalamus inflammation[40,42], which occurs before significant body weight gain occurs. These studies also showed that this hypothalamic inflammation is higher when metabolic

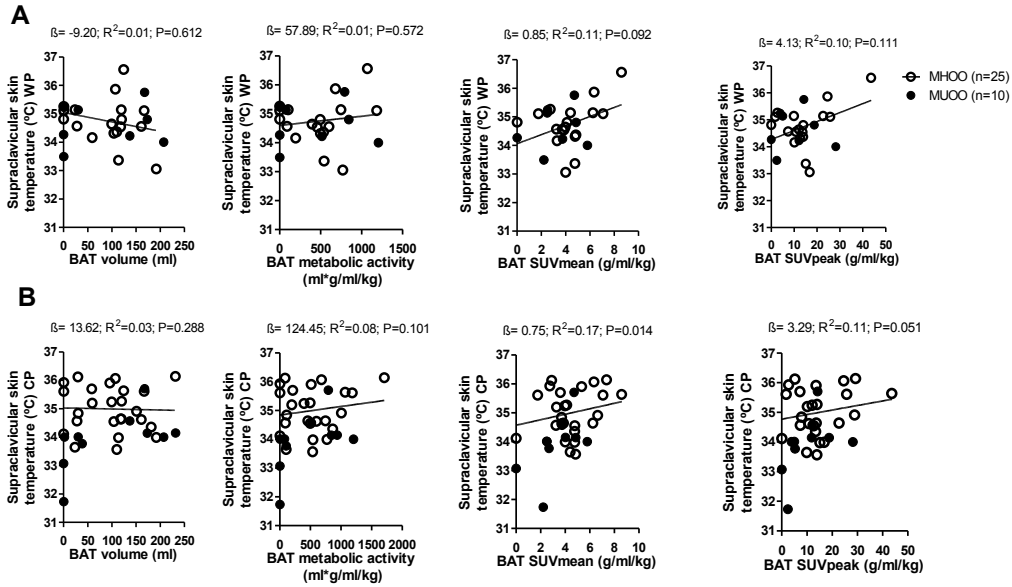

Figure 7. Association of supraclavicular skin temperature at the end of the warm (A) and cold period (B) with brown adipose tissue (BAT) volume, metabolic activity, standardized uptake value (SUV) mean and SUVpeak, respectively, in metabolically healthy but overweight-obese (MHOO) and metabolically unhealthy but overweight-obese (MUOO) participant. P values of linear regression adjusting by sex. CP: cold period; SUV: standardized uptake value; WP: warm period.

syndrome is presented, although further evidence is needed. Considering the hypothesis of the intraindividual variability of TRPM8 in the skin and the hypothalamic inflammation, it is plausible that MHOO individuals are more sensitive to the perception of cold because they have a higher amount of TRPM8 in the skin and/or less hypothalamic inflammation, and therefore their BAT works different. Maybe, the fact that a person shivers faster and feel colder in a cold environment is an indirect marker of the metabolic status, yet this hypothesis should be confirmed.

On the other hand, Ferrannini et al.[43] recently showed that type 2 diabetes participants had lower blood flow in the subcutaneous adipose tissue of the abdomen, whereas the 18F-FDG uptake by skeletal muscle was similar to non-diabetic participants. In addition, Summer et al.[44] reported that WAT of obese participants has a reduced capillarization. Additionally, Rosdahl et al.[45] showed that during an insulin clamp the increased blood flow of the forearm was due to the vasodilation of the adipose tissue rather than the internalization of the glucose in the skeletal muscle. Based on these findings, MHOO might have better adipose tissue blood flow in comparison to MUOO group. This fact, could explain the differences in the thermal sensations, because the MHOO group might have the possibility to perform a higher and faster peripheral vasoconstriction of the vascularization in their subcutaneous adipose tissue. However, our data showed no differences between groups on skin temperature responses (Figure 6).

Nevertheless, we observed that supraclavicular skin temperature upon a cold exposure was higher in MHOO compared to MUOO. Supraclavicular skin is an indirect marker of BAT activity , yet the fossae is composed by other tissues as vessels, skeletal muscles or adipose tissue[46], which probably are contributing to the skin temperature. However, the fact that this temperature is not the same between both groups could be explained because the contribution of these tissues to the

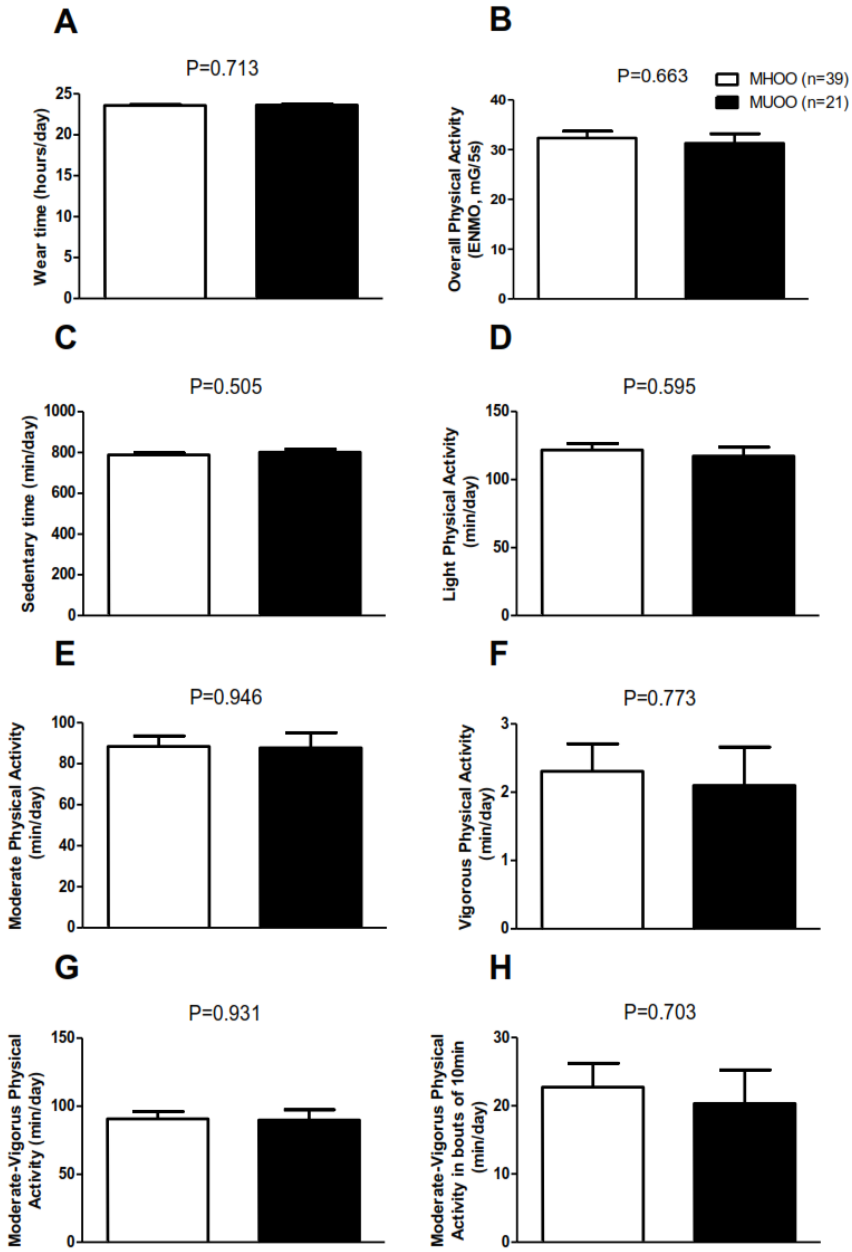

Figure 8: Physical activity levels in metabolically healthy but overweight-obese (MHOO) (n=39) and metabolically unhealthy but overweight-obese (MUOO) (n=21) participants. A. presents the differences in wear time of the accelerometer. B. presents the overall activity, whereas C, D, E, F, G and H, present the differences in sedentary time, low, moderate, vigorous, moderate-vigorous and moderate-vigorous in bout of ≥10 min, respectively. Dara are adjusted means and standard error. P values from analysis of covariance adjusting by sex. ENMO: Euclidean norm minus one.

supraclavicular skin temperature might be different between phenotypes. Sarasniemi et al.[47] showed that in obese participants the fat layer of the supraclavicular zone was an important covariate in the determination of the supraclavicular skin temperautre, and Gatidis et al.[48] observed that supraclavicular fat layer was positively

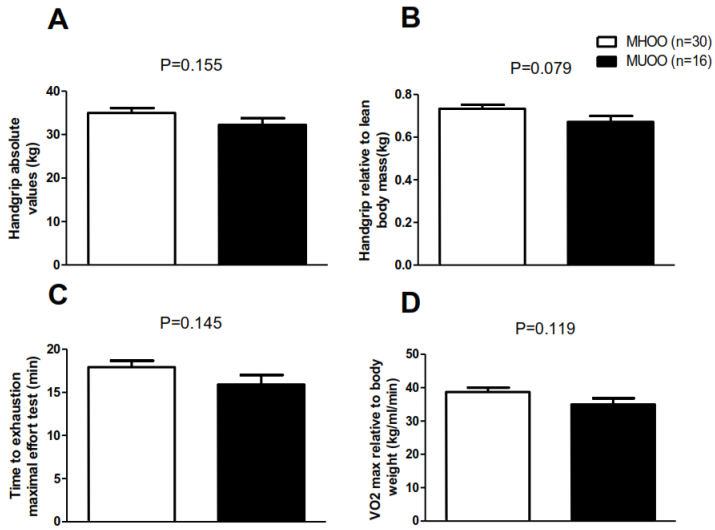

Figure 9: Muscular and cardiorespiratory fitness (CRF) levels in metabolically healthy but overweight-obese (MHOO) (n=30) and metabolically unhealthy but overweight-obese (MUOO) (n=16) participants. A. presents the differences between handgrip strength, whereas B. presents the differences between handgrip strength relative to lean body mass. C and D present the differences in time to exhaustion and maximum volume of oxygen (VO2max) relative to body weight. Data are adjusted means and standard error. P values from analysis of covariance adjusting by sex.

associated with BMI. Both MHOO and MUOO had similar BMI and it is likely that the supraclavicular fat layer was also similar. Further studies are needed to elucidate what is actually measuring the supraclavicular skin temperature.

PA and fitness levels

Recently, Ortega et al. [39] showed that MHO have higher levels of PA and that they spend lower time at sedentary activities, as well as they had higher levels of VO2max, whereas there are no differences in muscular strength between MHO and MUO. In contrast, we did not observe between group differences in PA and fitness levels. Of note is that the participants included in the systematic review of Ortega et al. [39] were adult people (>45 years old), whereas our participants were younger (18-25 years old). Maybe PA plays a more important role in older individuals who have been exposed for a longer period of time to obesity. Nevertheless, finding strategies to improve the metabolic profile of the people by exercise interventions is of world interest.

Limitations

This is an observational study, thus we cannot determine any cause-effect. BAT quantification was performed by a static 18F-FDG-PET/CT scan, therefore replication studies with other tracers are needed [49]. Moreover, our participants were exposed to different water temperatures; therefore, future studies using fixed cooling protocol are needed.

CONCLUSIONS

MHOO showed higher levels of BAT volume and activity, as well as glucose uptake in the dorsocervical area. MHOO individuals also perceived colder their bodies, feet and hands one month before the enrolment, as well as upon acute cold exposure in comparison to MUOO. Moreover, the levels of PA and fitness were similar between phenotypes. These results suggest that the MHOO group have a better thermoregulatory response in comparison to MUOO group. Further studies with specific interventions are needed.

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General Discussion

An integrative discussion of the International Doctoral Thesis

CHAPTER 15

GENERAL DISCUSSION

Since it is known that humans present metabolic active BAT, numerous studies have been focused in elucidate its role as possible therapy to combat adiposity, type 2 diabetes or CVD[1–4]. The amount of BAT in the human body is relatively low[5] in proportion to other tissues, and several studies have shown that the contribution of BAT in the human energy metabolism is almost negligible[6,7]. Alternatively, we cannot preclude the possibility that human BAT has a role as endocrine, paracrine or autocrine[8] organ, influencing the human metabolism.

Moreover, BAT has a high vascularization [9,10] and the capacity to generate heat[11]; thus this tissue could have a key role in the human thermoregulatory system as is the case in mice. Exercise is known for being one of the most powerful treatment for all diseases[12]. Moreover, exercise has a positive impact on the skeletal muscle function[13] and could eventually have an impact on BAT. Several studies have postulated that BAT could serve as a metabolic 'sink' that internalises glucose thereby correcting hyperglycaemia and improving insulin resistance. Therefore, unravelling the implication of this tissue in a clinical population who present metabolic syndrome will improve our knowledge about the role of BAT in humans. Nevertheless, skeletal muscle is representing a great proportion of tissue in the human body, and has shown to have an important role in the improvement of the insulin resistance[14], as well as in the energy metabolism or temperature regulation among other features.

To gain more insights into these questions, we investigate the current methodology for both skin temperature (**chapters 2, 3** and **4**) and BAT assessment (**chapters 5, 6, 7** and **8**). In the **chapter 9**, we show that the ambient temperature that a person is exposed could be measured by 1 or 2 iButtons, and that the peripheral vasoconstrictions/vasodilatations are related with a possible BAT activation or inhibition (**chapter 10**). Moreover, the **chapter 11** shows that women feel colder in comparison to men after the ingestion of a meal intake, despite their skin was warmer. In the **chapter 12**, we show that the glucose uptake in the dorsocervical area is higher in comparison to other areas, whereas in **chapter 13** we observe that handgrip strength is positively associated with BAT activity. Furthermore, **chapter 14** shows that the MHOO phenotype had a higher BAT volume and activity, although they spent slightly less time exposed to cold, and perceived colder the temperature upon an acute and chronic cold exposure in comparison to MUOO.

From this International Doctoral Thesis, various new insights on the thermoregulatory system, BAT and exercise have been arisen which will be discussed and interpreted in this chapter. In addition, possible clinical implications derived from this thesis and future directions are be addressed.

SKIN TEMPERATURE ASSESSEMENT

Nowadays, there is no gold standard for skin temperature assessment. The interest of the scientific community on this outcome is increasing because it is cheap and easy to measure, and it gives information about how the body reacts to different stimulus. The most used instruments for measuring skin temperature are thermocouples, IRT and iButtons. The later are small devices that in lab conditions show accurate measurements [15], yet in **chapter 4** we show that iButtons measure by both sides and this "issue" has not been addressed before. Moreover, we show that isolating one side of the iButtons we are able to obtain more accurate skin temperature assessment. Despite this fact, one of the biggest limitation of iButtons is that the current models are not wireless, so it is impossible to observe how the body reacts to different stimuli in vivo. The company currently offers a free open software (OWV), which has several limitations for instance OWV does not offer the possibility to analyse the amount of data generated by iButtons (**chapter 2**). In the ACTIBATE study, we

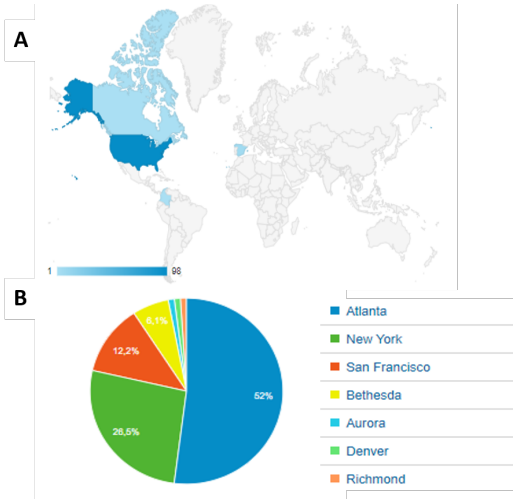

Figure 1. Demographics statistics obtained by google analytics about the countries that have downloaded Temperatus ® software (A). Downloaded percentages classified by USA's cities (B).

managed hundreds of iButtons every day. To manage all this information without making mistakes with the OWV software was almost impossible. Therefore, we develop the Temperatus® software (**chapter 2**), which is freely available http://profith.ugr.es/temperatus?lang=en. Temperatus® software has been downloaded from more than 95 different locations along all over the world in less than one year, being United States of America (USA) the country with the higher amount of users (Figure 1A and B). Within USA, the main downloaded has been performed from Atlanta or New York where the headquarters of the company owned the iButtons (Maxim Integrated) are located (Figure 1B). One of the reasons about why there is no gold standard for measuring skin temperature is the discrepancies that exist regarding the anatomical points and equations selected for measuring skin temperature. In **chapter 3**, we show that the equation selected strongly influences the final outcome. We show that there are 4 parameters, which are accepted within the scientific community of the thermophysiology, which should be reported in all the studies focus in the thermoregulatory responses. The skin temperature parameters are the following; mean, proximal, distal skin temperatures and the gradient forearm-fingertip, as a proxy of peripheral vasoconstriction or vasodilatation[16]. Reporting all this data, allow to the researchers to show the whole body thermoregulatory response to the stimiuli. In addition, Boon et al.[17] showed, by first time, that supraclavicular skin temperature measured by an iButton could be an indirect marker of BAT activity, as well as van der Lans et al.[18] or Law et al.[19]. Curiously, supraclavicular skin temperature did not decrease after a cold exposure as we observed in **chapter 3, 4, 8, 12** or slightly increased after a meal intake being higher in women than men (**chapter 11**). Several studies suggested that this no decrease after a cold exposure in the supraclavicular skin temperature in comparison to other skin temperature regions (which decreased after cold), is reflecting BAT activation[17,18,20]. Nevertheless, the studies that have performed the validation of skin temperature as a proxy of BAT activity have showed weak correlation coefficients, or they did not take into account some limitations. In **chapter 8**, we show no association between supraclavicular skin temperature measured by iButtons and IRT with 18F-FDG uptake by BAT. Therefore, our data suggests that supraclavicular skin temperature could not be a valid proxy of BAT volume and activity in young adults.

In 1987, Kellman et al.[21] performed an outstanding study where they analyzed the tissue-composition of the supraclavicular fossa in humans. They found that the supraclavicular fossa is composed by some fat, lymph nodes, and the posterior lunge apex, although the main components are subclavian vessels, brachial plexus, omohyoid and scalene muscles. Recently, Blondin et al.[6] observed that deep and cervical muscles had a higher 18F-FDG uptake in comparison to other skeletal muscles after a cold exposure. U Din et al.[7] using 15O-O2-PET/CT scan demonstrated that deep and cervical muscles were the main contributors of the increase of energy expenditure upon a cold exposure in humans. This evidence showed that skeletal muscles are contributing to thermogenesis, and in higher proportion that BAT in humans. The main physiological role of these tissues could be to

warm-up the temperature of the blood that is around the neck in order to control the drop of temperature in the brain and cerebellum upon a cold exposure[22–24]. Moreover, supraclavicular fat layer is an insulation tissue that depending on its own thickness seems to mediate the measurement of supraclavicular skin temperature[25]. Therefore, supraclavicular skin temperature measured by iButtons or IRT upon a cold exposure represent a sum of different tissue's features (i.e. thermogenic activity of skeletal muscles and BAT, temperature of vessels, insulation of supraclavicular fat layer thickness, etc.). Nowadays, the current techniques are not able to distinguish which the heat production of every tissue is. Thus, it is not correct to assume that the no decrease of supraclavicular skin temperature by a cold exposure is caused solely by BAT activation. Furthermore, we show in **chapter 12** that dorsocervical skin temperature does not decrease after a cold exposure. These findings suggest that these anatomical zones (supraclavicular and dorsocervical areas, which are not included in the current equations to estimate mean or proximal skin temperature) are contributing to maintain the core body temperature upon a cold exposure. These zones should be taken into account in future equations for the estimation of mean and proximal skin temperature, because they are representing part of a physiological response that is happening in the body.

BROWN ADIPOSE TISSUE METHODOLOGY

One of the biggest limitations in the field of human BAT quantification is the huge discrepancy about the cooling protocols used to stimulate BAT. In brief, there are two types of cooling protocols, fixed vs. personalized temperatures[26–28]. Both protocols have their own limitations and the election of one type or the other depends on the scientific questions that need to be answered. The main aim of the personalized cooling protocol is to reduce the 18F-FDG uptake by skeletal muscles in order to reduce the "stealing phenomena"[29,30], yet it is possible that these phenomena is not happening in humans. There are several reviews that have addressed this issue[26,31], and a panel of experts (BARCIST 1.0) recognized both protocols as valid tools to quantify BAT [27]. In **chapter 5**, we discuss the cooling protocol that we have used in the ACTIBATE study[32]. The novelty of this protocol is that we calculated the shivering threshold test in an independent day of the quantification of BAT. With this protocol we observed similar levels of BAT SUVmax in comparison to other studies[33,34] that performed the shivering threshold test and the personalized cooling protocol in the same day. In **chapter 6,** we detect that every study estimate BAT using different combinations of thresholds. Thus, we decided to compare the three most used combination of thresholds in literature with the BARCIST 1.0 criteria[27]. We observe that the most comparable BAT outcome across criteria is the SUVmax or SUVpeak, whereas the less comparable is BAT volume. Furthermore, **chapter 6** describes that the most used combination of thresholds for BAT quantification is the fixed SUV threshold of 2.0 g/ml and the HU range from -50 to -250, which was used by first time by Cypess et al. in 2009[35]. **Chapter 7** shows that the most used range of HU (from -50 to -250) for BAT quantification is missing approximately more than the 40% of the total BAT volume of a person, which represent around the 43.2% of the total BAT metabolic activity. These novel findings suggest that more than 27 studies (see table 2 of **chapter 6**) are missing more than the 40% of the total BAT metabolic activity of their cohorts. We show that using the upper threshold of -50 instead of -10 is affecting several BAT outcomes, for instance the mean HU in the BAT depots, which U Din et al.[36] suggested that is negatively related with adiposity. To the light of these findings, whether those studies that have used the most common combination of threshold to quantify BAT, they re-analyse their data of BAT, probably some of their conclusions might change. Moreover, in the **Chapter 7** we observe that modifying the SUV threshold could affect in a higher proportion the outcomes of BAT volume or activity in comparison to modifying the HU range.

Chapter 7 also describes that analysing the amount of BAT in every single range of HU in a statics 18F-FDG-PET/CT scan allows obtaining valuable information about the distribution of BAT radiodensity. Radiodensity is used as a proxy of the fat content in the tissue[36], although we acknowledge that we are missing an important part of the BAT radiodensity since we do not have an 18F-FDG-PET/CT scan in thermoneutral conditions. The **chapter 14** shows that MHOO individuals have a higher distribution of BAT radiodensity from -10 to -100 HU in comparison to MUOO, and we observe in **chapter 7** that the distribution of BAT radiodensity was different in men vs. women.

THERMOREGULATION: SKIN TEMPERATURE

In the present International Doctoral Thesis we developed the Temperatus® software, which allowed us to perform detailed analyses about the kinetics of skin temperature in response to different stimulus. For instance in **chapter 11**, we study that a standardized liquid meal intake induced a peripheral vasoconstriction during the first hour, which means a redistribution of the blood flow from the peripheral part of the body to the proximal part. We observe an increase in the mean, proximal and distal skin temperatures as well as peripheral vasodilation. We also show that this phenomenon is independent of the individual's body composition or the menstrual cycle. Moreover, in **chapter 14** we observe that kinetics of skin temperature in response to this standardized meal intake are similar between the phenotype of MHOO vs. MUOO, whereas upon the cold exposure are slightly different only in the supraclavicular skin temperature. These results support the idea that skin temperature should be analysed as kinetics and by mean, proximal, distal and the gradient forearm-fingertip, because all these data allow to perform a deeper interpretation of the thermorreguralory response of the body. Those studies that analyse only the average of five minutes before and after the stimuli they might be missing a valuable information about the response of the thermoregulatory system of their participants.

A big "problem" for human BAT quantification is the outdoor temperature or seasonal variation[37]. In **chapter 9**, we propose that WT and Personal-ET measured by iButtons one week before BAT quantification is able to measure the real temperature that the participants are exposed during this time period. We observe that both outcomes are negatively and significant associated with 18F-FDG uptake by BAT. Curiously, we show that WT and Personal-ET are also negatively related with 18F-FDG uptake by skeletal muscles, which suggest that ambient exposure is affecting more a possible translocation of GLUT4[1,38] in all tissues than only a specific tissue (BAT). Therefore, future studies analysing whether outdoor temperature or seasonal variation is affecting the tissues measured by other tracers such as 18F-FTHA or 11C-acetate are needed. Furthermore, in **chapter 10**, we show that BAT is mediating the relationship between Personal-ET with WT. We observe that whether two participants are exposed to the same Personal-ET, the participant who has higher levels of BAT, his/her WT will be slightly higher at the end of 7 days. This finding support the idea that maybe WT is not a good marker of the real exposition of the participants and maybe Personal-ET should be used as unique measurement. Moreover, WT is used in the field of the chronobiology as a marker of circadian rhythms[39–41]. We have found that WT is affected by ambient exposure, therefore those studies that are measuring WT in different months; they should take into account that the ambient exposure of every participant could affect the parameters of WT. There is evidence that suggest that WT could be used as indirect proxy of peripheral vasoconstriction and vasodilatations[16]. In the **chapter 10** we show a relationship between peripheral vasoconstriction /vasodilation with the levels of BAT. In other words, those participants who are able to perform a higher peripheral vasoconstriction have also higher levels of BAT volume, higher metabolic activity and higher SUVpeak. In warm ambient we observe the opposite. These results suggest that might be a

relationship between the skin blood flow and the activation of BAT. Why are there participants that to the same ambient exposure have a different physiological response? This question remains unaddressed.

THERMOREGULATION: THERMAL PERCEPTION

Cold is a stimuli that is not comfortable at all for humans[42]. In **chapter 5**, we show that our participants perceive colder during the shivering threshold test in comparison to the personalized cooling protocol, which it is logical since the shivering threshold test was designed to induce shivering and the personalized cooling protocol was not. In **chapter 11**, we observe that women perceive a little bit colder the temperature of the room, which is supposed to be thermoneutral, which concur with early studies [43,44]. Moreover, we do not find any relationship between the changes in the thermal perception upon a meal intake with the changes in skin temperature. This lack of association could be based on the fact that thermal perception and skin temperature responses seem to be integrated in different part of the hypothalamus[45]. **Chapter 14** shows that despite the fact that MHOO and MUOO groups are exposed to similar ambient exposure, the MHOO group perceive colder the temperature in their body, feet and hands in comparison to MUOO. Furthermore, MHOO individuals perceive higher the shivering and the cold in comparison to MUOO. Interestingly, the water temperature of the cooling vest of the MHOO group was slightly higher in comparison to MUOO group, yet the MHOO group had higher levels of BAT. In mice, several studies showed that the cold-tolerance depend on the amount of BAT[46,47], but maybe in humans is not like that. Maybe the differences that we observed in the thermal perception and levels of BAT in the MHOO and MUOO phenotypes is suggesting that those participants who are able to start shivering faster is related with a healthier metabolic profile. Recently, Jais and Brüning [48] highlighted that hypothalamic inflammation has been linked to the development and progression of obesity. The thermal perceptions are integrated in the hypothalamus, and therefore, these differences in perception could be explained because the MHOO group has a higher resistance to a neuronal inflammation in comparison to MUOO. The possible reduced neural inflammation in MHOO individuals could explain a better perception and better physiological responses in comparison to their counterparts, who should have a higher neural inflammation. This higher neural inflammation in the MUOO group, could affect the afferent and efferent pathways between the perceptions in the skin and the physiological responses to the different stimulus, making this phenotype weaker upon a cold exposure. However, these assumptions should be addressed in the near future.

DORSOCERVICAL GLUCOSE UPTAKE

WAT in mice shows different levels of browning depending on the anatomical region, for instance inguinal vs. epidymal WAT[49] upon different stimulus. Most of the human studies published to the date have performed the biopsies of the WAT in the abdomen or in the thigh[50,51]. Some studies have failed to find browning after a cold exposure in the SAT in the abdomen[52]. In **chapter 12**, we show that the skin temperature of the abdomen decreases upon a cold exposure in young lean men. As in mice, it could be that the levels of browning in the SAT of the abdomen might be lower in comparison to other SAT zones. From an evolutionary perspective [53,54], the SAT of the dorsocervical area would be an alternative zone for performing biopsies because newborns showed subcutaneous BAT[54], that they lose when they grow. **Chapter 12** describes that the 18F-FDG uptake is higher in the dorsocervical area in comparison to the SAT of the tricipital zone. Moreover, the 18F-FDG uptake by the SAT in the dorsocervical area correlate with the 18F-FDG uptake by BAT in the supraclavicular zone, whereas the 18F-FDG uptake by the SAT of the triceps does not. Moreover, **chapter 14** shows that 18F-FDG uptake in the

dorsocervical area is higher in MHOO, whereas the 18F-FDG uptake in the SAT of the tricipital area was the same between MHOO and MUOO. We also observe that the skin temperature in the dorsocervical area does not decrease after the cold exposure, where this high 18F-FDG uptake is located (**chapter 12**). Recently, U Din et al.[55] showed that the oxygen consumption in the SAT of the dorsocervical area increase after meal intake, as well as the blood flow or the 18F-FTHA uptake. They did not find these changes in the SAT of the tricipital area, although they argued that this could be an artefact due to the compression of supine body. However, we think that these compressions of the body could not explain the differences in 18F-FDG uptake that we observed between the SAT of the dorsocervical and tricipital area. Therefore, based on these findings, there is an imperial need to perform biopsies of the SAT of the dorsocervical area and studying the molecular composition of this tissue.

EXERCISE: PHYSICAL FITNESS

Recently, we observed that the levels of PA objectively measured were not associated with the levels of BAT in young adults[56]. In addition, **Chapter 13** shows a positive and significant association between HG strength relative to LBM with BAT activity, whereas there was no association with CRF. Moreover, **chapter 14** describes that there were no differences between the levels of muscular strength, CRF or PA levels in the phenotypes of MHO vs. MUO. To the light of these results and previous evidence, we suggest that future exercise interventions focus on the resistance training[57] more than endurance training are needed, because the physiological adaptations that both stimulus can induced in the human metabolism are different.

MAIN LIMITATIONS

The present International Doctoral Thesis presents several limitations that have been acknowledge during every chapter. Nevertheless, one of the biggest limitation is that we measured BAT with a glucose analogue (18F-FDG) and we know that brown adipocytes consume more fatty acids than glucose[58]. Therefore, replicating these findings with other nuclear medicine techniques for BAT quantification is needed[26,59]. We also quantify skeletal muscle 18F-FDG uptake and we know that this tracer, could not be the best tecnhique for measuring the metabolic activity of this tissue. Moreover, we used iButtons that in **chapter 4** we show that are measuring by both sides and we show that polyethylene could be a good solution for this problem. We realized of that issue after performing the data collection, however in all of our analysis we have tried to adjust the analysis by ambient or water temperature when it was needed. We did not measure the level of clothing of our individuals during the day-living measurement of WT and Personal-ET. We used the personalized cooling protocol, without using electromyography for detecting shivering; therefore the results of the present thesis should be interpreted with caution. Lastly, we did not measured supraclavicular fat layer, which Sarasniemi et al.[60] showed that is mediating the relationship between supraclavicular skin temperature and BAT temperature.

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Concluding Remarks and Future Perspectives

The main conclusions of this International Doctoral Thesis are: (i) we improve skin temperature and BAT quantification with 18F-FDG-PET/CT scans for human studies (Part 1 and Part 2); (ii) we observe that BAT could be playing a role in the human thermoregulatory system (Part 3). Lastly, we found that physical fitness could be related with BAT, and we observe that the levels of BAT are higher in the phenotype of MHOO vs. MUOO individuals.

MAIN CONCLUSIONS

The main conclusions of the present International Doctoral Thesis are:

Part 1: The importance of understanding the instruments: skin temperature assessment

Chapter 2: To our knowledge, the Temperatus® software is the unique alternative to the OWV software to programme, download, and the unique software that allows users to analyse massive amounts of data coming from different iButtons.

Chapter 3: We detect differences in skin temperature across the studied equations in both warm and cold room conditions. Based on these findings, we suggest a set of 19 ibuttons to estimate mean, proximal, and distal skin temperatures as well as body temperature gradients

Chapter 4: iButtons are registering temperature by both sides, and the output seems to be an average of both sides. We propose and validate an isolated alternative based on polyethylene. With this isolation, we obtain more accurate measurements of skin temperature in both warm and cold conditions.

Part 2: The importance of understanding the instruments: brown adipose tissue methodology

Chapter 5: Our cooling protocol is able to activate BAT in young adults. The novelty of our protocol resides in the fact that we applied just 2 hours of personalized cold exposure prior to PET/CT scan while the shivering threshold was determined on a separate day (48-72 hours before).

Chapter 6: BAT volume and activity, as determined by 18F-FDG PET/CT, highly depends on the quantification criteria used. According to our findings, when following a personalized cooling protocol, SUVpeak and SUVmax are the most consistent markers of maximal BAT activity independent of the HU and SUV threshold used, which may therefore facilitate comparisons across studies.

Chapter 7: SUV thresholds determines BAT quantification more than HU thresholds. It is important to include the range from -10 to -50 HU because it contains a huge amount of BAT volume (43.2%), which represents 41.4% of the total BAT metabolic activity in our study.

Part 3: Brown adipose tissue and thermoregulation

Chapter 8: iButtons and the mean measurement of IRT are not comparable. Moreover, there is no association between supraclavicular skin temperature measured by iButtons or by IRT with BAT activity, as well as with 18F-FDG uptake by skeletal muscle

Chapter 9: WT and Personal-ET are negatively associated with BAT and skeletal muscle 18F-FDG uptake. Moreover, the number of hours per day exposed to different ranges of cold temperatures are differently correlated with BAT and skeletal muscles 18F-FDG uptake. We show that during daily living conditions 18F-FDG uptake by BAT and skeletal muscles seem to be involved in the thermoregulatory system hierarchically, although the correlation coefficients are weak.

Chapter 10: BAT volume and metabolic activity mediate the relationship between Personal-ET and WT. Moreover, individuals were exposed to lower environmental temperatures and at the same time had lower WT, concomitantly had higher BAT volume.

Chapter 11: A standardized and individualized liquid meal intake increases the skin

temperature in young adults. Women present a higher increase of skin temperature parameters than men in response to a meal intake, regardless of their body composition and menstrual cycle.

Part 4: Clinical Implication

Chapter 12: 18F-FDG uptake by the SAT of the dorsocervical area is higher in comparison to other SAT area. The observed high glucose uptake in this area could be iBAT.
Chapter 13: There is a positive association between HG relative to LBM strength with 18F-FDG uptake by BAT activity (SUVmean and SUVpeak), whereas there is no association between CRF and BAT-related outcomes in young adults.
Chapter 14: MHOO phenotype have higher levels of BAT volume and activity in all ranges of HU in comparison to MUOO, as well as higher levels of 18F-FDG uptake in the dorsocervical. The MHOO perceive colder their bodies, feet and hands in comparison to MUOO. Moreover, the levels of PA and cardiorespiratory fitness are similar between phenotypes.

FUTURE PERSPECTIVES

- Mean, proximal and distal skin temperatures should be improved introducing other anatomical areas such as supraclavicular or dorsocervical region.
- There is a need in the field of using new or alternative devices to the iButtons. These devices should be able for measuring only for one side and measuring wireless.
- The fact that supraclavicular skin temperature did not decrease or in some occasions increased upon a cold exposure need further investigations. Why does it happen? Which are the main tissues involved in this no decrease? It seems that BAT is not the only tissue involved and therefore this question should be answered.
- Further studies controlling the clothing of the individuals, as well as the exposure of these people to the ambient exposure is of interests.
- Future studies including brain analyses measured by functional magnetic resonance imaging and skin biopsies are needed in order to elucidate the role of human BAT on the thermal perceptions and in the thermoregulatory responses.
- Biopsies of the SAT of the dorsocervical area is of imperial need.
- Muscle strength training could be a potential BAT activator.
- Further studies focusing in the role of muscle strength training on the browning levels are of interest in the field.
- Nowadays, why there are obese people with a healthy cardiovascular profile remains a mystery. The differences in BAT levels as well as in the thermal perceptions suggest a role of the hypothalamus, but this hypothesis need to be studied.

Anexes

PAPERS DERIVED FROM THE THESIS

1. Ruiz JR, Martinez-Tellez B, Sanchez-Delgado G, Aguilera CM, Gil A. Regulation of energy balance by brown adipose tissue: at least three potential roles for physical activity. Br J Sports Med 2015; 0:2014–6.

2. Martinez-Tellez B, Sanchez-Delgado G, Boon MR, Rensen PCN, Ruiz JR. Activation and quantification of human brown adipose tissue: methodological considerations for between studies comparisons: Comment on: Hot heads & cool bodies: The conundrums of human BAT activity research. Eur J Intern Med 2017; 40:e19-e21.

3. Ruiz JR, Martinez-Tellez B, Sanchez-Delgado G, Osuna-Prieto FJ, Rensen PCN, Boon MR. Role of Human Brown Fat in Obesity, Metabolism and Cardiovascular Disease: Strategies to Turn up the Heat. Prog Cardiovasc Dis 2018; 61(2):232-245.

4. Martinez-Tellez B*, Quesada-Aranda A*, Sanchez-Delgado G, Fernández-Luna JM, Ruiz JR. Temperatus® software: a new tool to efficiently manage the massive information generated by iButtons. Submitted.

5. Martinez-Tellez B, Sanchez-Delgado G, Acosta FM, Alcantara JMA, Boon MR, Rensen PCN, Ruiz JR. 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):10530.

6. Martinez-Tellez B, Acosta FM, Sanchez-Delgado G, Rensen PCN, Ruiz JR. A methodological approach to improve skin temperature measurement using iButtons in human cold-induced studies. In preparation.

7. Martinez-Tellez B, Sanchez-Delgado G, Garcia-Rivero Y, Alcantara JM, Martinez-Avila WD, Muñoz-Hernandez V, Olza J, Boon MR, Rensen PCN, Llamas JM, Ruiz JR. A new personalised cooling protocol to activate brown adipose tissue in young adults. Front Physiol 2017; 8:863.

8. Martinez-Tellez B*, Nahon KJ*, Sanchez-Delgado G, Abreu-Viera G, Llamas-Elvira JM, van Velden FHP, Arias-Bouda L, Rensen PCN, Boon MR, Ruiz JR. The impact of using BARCIST 1.0 criteria on quantification of BAT volume and activity in three independent cohorts of adults. Sci Rep 2018; 8(1):8567.

9. Martinez-Tellez B, Sanchez-Delgado G, Boon MR, Rensen PCN, Llamas-Elvira JM, Ruiz JR. Distribution of human brown adipose tissue radio-density in young adults. In preparation.

10. Martinez-Tellez B*, Perez-Bey A*, Acosta FM, Sanchez-Delgado G, Corral-Pérez J, Amaro-Gahete FJ, Alcantara JMA, Castro-Piñero J, Jimenez-Pavon D, Llamas-Elvira JM, Ruiz JR. Concurrent validity of supraclavicular skin temperature measured with iButtons and infrared thermography as a surrogate marker of brown adipose tissue. In preparation.

11. Martinez-Tellez B*, Xu H*, Sanchez-Delgado G, Acosta FM, Rensen PCN, Llamas-Elvira JM, Ruiz JR. Association of wrist and ambient temperature with cold-induced Brown adipose tissue and skeletal muscle 18F-FDG uptake in young adults. Am J Physiol Regul Integr Comp Phsiol 2018; In press

12. Martinez-Tellez B*, Adelantado-Renau M*, Acosta FM, Sanchez Delgado G, Martinez-Nicolas A, Llamas-Elvira JM, Boon MR, Martinez-Vizcaino V, Ruiz JR. The mediator role of Brown adipose tissue and skeletal muscle in the thermoregulatory system of young adults. Submitted.

13. Martinez-Tellez B*, Ortiz-Alvarez L*, Sanchez-Delgado G, Acosta FM, Muñoz-Hernandez V, Martinez-Avila W, Merchan-Ramirez E, Contreras-Gomez M, Gil A, Labayen I, Ruiz JR. Skin temperature response to a liquid meal intake is different in men than in women. Clin Nutr 2018; pii: S0261-5614(18)30212-7.

14. Martinez-Tellez B, Sanchez Delgado G, Alcantara JMA, Acosta FM, Amaro-Gahete

FJ, Osuna-Prieto FJ, Perez-Bey A, Jimenez-Pavon D, Llamas-Elvira JM, Gil A, Aguilera CM, Rensen PCN, Ruiz JR. Evidence of metabolically active interscapular brown adipose tissue depots in adults. Submitted.

15. Martinez-Tellez B, Sanchez Delgado G, Amaro-Gahete FJ, Alcantara JMA, Acosta FM, Ruiz JR. Associations between cardiorespiratory and muscular fitness with 18F-fluorodeoxyglucose uptake by brown adipose tissue and skeletal muscle in young adults. In preparation.

16. Martinez-Tellez B*, Sanchez Delgado G*, Acosta FM, Amaro-Gahete FJ, Alcantara JMA, Ruiz JR. Distribution of brown adipose tissue, the thermoregulatory system and, physical activity and fitness in metabolically healthy but overweight-obese adults: a case control study. In preparation.

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Short- Curriculum Vitae

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2. Current Affiliation

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3. Scientific and clinical education

06/2018 – 08/2018: Internship of 3 months at LUMC (the Netherlands)
03/2018 – 03/2018: Internship of 2 weeks at National Institutes of Health (NIH, USA).
07/2017 – 09/2017: Internship of 1.5 months at LUMC (the Netherlands).
06/2016 - 09/2016: Internship of 3 months at LUMC (the Netherlands).
09/2014 – 11/2018: PhD student of Biomedical Sciences, University of Granada.
09/2014 – 09/2015: Master in Physical Activity and Health, University of Granada.
10/2013 – 07/2014: Master in Physical Education, University of Granada.
09/2009 – 09/2013: 5-year bachelor degree in Sport Sciences, University of Granada.

4. Research experience

09/2014 – present	-Project manager 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 Financing: Spanish Ministry €175,000; others: €150,000
07/2014 – 07/2015	-Project manager at Dept. Physical Education and Sport, UGR. Project: Effect of a protein shake in muscle damage and recovery in triathletes. Principal Investigator: Jonatan R. Ruiz Financing: Puleva Lactalis S.L. €190,000
09/2013 – 07/2014:	-Student assistant at Dept. Physical Education and Sport, UGR Project: Proposal of a Field-Based Physical Fitness-Test Battery in Preschool Children: The PREFIT Battery. Supervisors: Francisco B. Ortega and Jonatan R. Ruiz Financing: Spanish Ministry €10,000

5. Management activities

11/2016 – 12/2016	Organizer committee of ACTIBATE symposium, Granada, Spain.
01/2015 – present	Co-developer of the Temperatus® software to manage iButtons data.
09/2014 – 12/2014	Organizer committee of the Exernet Symposium "Exercise is medicine" at University of Granada.
07/2013 – 10/2013	Volunteering for organizer committee the international congress of Nutrition.

6. Courses and extracurricular activities

02/2017 – 02/2017	Course "Multiple Linear Regression"
11/2016 – 12/2016	ACTIBATE symposium "Preliminary Results", Univ. Granada, Spain
04/2015 – 05/2014	How to perform a Meta-analysis
02/2015 – 02/2015	Course "Which are the relationship between human organs?
10/2014 – 10/2014	Course "How to use Mendeley desktop"
06/2014 – 07/2014	Course "Scientific writing: How to write a paper"

7. Personal grants

2018: Postdoctoral Fellows ENERGISE consortium: November 2018-2019 (1 year). (€70.000).
2018: Short internship grant: 3 months to stay in the LUMC "Albert Renold Travel Fellowship" funded by the European Foundation of the Study of Diabetes (EFSD) (€6,200).
2017: "Technical support and Management Staff" 12-months full time contract. Ref.:269, University of Granada (€24,000).
2016: Short internship grant: 3 months to visit LUMC (€3,000).

8. Educational activities

2016: Contribution to practicum at the Faculty of Physical Activity and Sports Sciences.

9. Conferences attended

Invited upon abstract submission

2018/10	PPTR congress, Split, Croatia. "The mediator role of Brown adipose tissue and skeletal muscle in the thermoregulatory system of young adults"
2018/05	ECO congress, Vienna, Austria. "Association of wrist and ambient temperature with cold-induced brown adipose tissue and skeletal muscle 18F-FDG uptake in young adults"
2017/05	EMBO workshop, Sitges, Spain. "Activation of brown adipose tissue in young adults: a novel personalized cooling protocol" (poster presentation).
2017/05	II (international congress of PhD students of University of Granada (JIFFI) congress, Granada, Spain. "An individualized cooling protocol to activate brown adipose tissue in young adults" (oral presentation).

2016/06 European Obesity Summit, Gothenburg, Sweden. "A cooling protocol prior PET/CT scan for BAT activity assessment: Preliminary results from the ACTIBATE study" (poster presentation).

2016/05 International congress of PhD students of University of Granada (JIFFI), Granada, Spain. "Skin temperature and subjective perception in a cooling test: Preliminary results from the ACTIBATE study" (oral presentation).

2014/11 Symposium Exernet: Exercise is Medicine, Granada, Spain. "Association of health-related physical fitness with total and central body fat in preschool children aged 3 to 5 years" (poster presentation).

PUBLICATIONS LIST

[1] Cadenas-Sanchez C, Sanchez-Delgado G, Martinez-Tellez B, Mora-Gonzalez J, España V, Ruiz JR, Ortega FB, Reliability and Validity of Different Models of TKK Hand Dynamometers **Am J Occup Ther** 2013: 1–9. (IF = 1.8).

[2] Segura-Díaz JM, Herrador-Colmenero M, Martínez-Téllez B, Chillón Garzón P. [Effect of precipitation and seasonal period on the patterns of commuting to school in children and adolescents from Granada]. **Nutr Hosp** 2014; 31:1264–72. (IF = 1.1).

[3] Cadenas-Sánchez C, Alcántara-Moral F, Sánchez-Delgado G, Mora-González J, Martínez-Téllez B, Herrador-Colmenero M, et al. Evaluación de la capacidad cardiorrespiratoria en niños de edad preescolar: adaptación del test de 20m de ida y vuelta**. Nutr Hosp** 2014; 30:1333–43. (IF = 1.1).

[4] Ortega FB, Cadenas-Sánchez C, Sánchez-Delgado G, Mora-González J, Martínez-Téllez B, Artero EG, et al. Systematic review and proposal of a field-based physical fitness-test battery in preschool children: the PREFIT battery. **Sports Med** 2015; 45:533–55. (IF = 5.6).

[5] Ruiz JR, Martinez-Tellez B, Sanchez-Delgado G, Aguilera CM, Gil A. Regulation of energy balance by brown adipose tissue: at least three potential roles for physical activity. **Br J Sports Med** 2015; 0:2014–6. (IF = 6.7).

[6] Ruiz JR, Sánchez-Delgado G, Martínez-Téllez B, Aguilera CM, Gil A. RE: Association between habitual physical activity and brown adipose tissue activity in individuals undergoing PET-CT scan. **Clin Endocrinol** (Oxf) 2015; 83(4):590-1 (IF = 3.5).

[7] Sanchez-Delgado G, Cadenas-Sanchez C, Mora-Gonzalez J, Martinez-Tellez B, Chillón P, Löf M, et al. Assessment of handgrip strength in preschool children aged 3 to 5 years. **J Hand Surg Eur** 2015; 40:966–72. (IF = 1.8).

[8] Sanchez-Delgado G, Martinez-Tellez B, Olza J, Aguilera CM, Gil A, Ruiz JR. Role of Exercise in the Activation of Brown Adipose Tissue. **Ann Nutr Metab** 2015; 21–32. (IF = 2.5).

[9] Sanchez-Delgado G, Martinez-Tellez B, Olza J, Aguilera CM, Labayen I, Ortega FB, et al. Activating brown adipose tissue through exercise (ACTIBATE) in young adults: Rationale, design and methodology. **Contemp Clin Trials** 2015; 45:416–25. (IF = 2.1).

[10] Cadenas-Sanchez C, Nyström CD, Sanchez-Delgado G, Martinez-Tellez B, Mora-Gonzalez J, Risinger AS, et al. Prevalence of overweight/obesity and fitness level in preschool children from the north compared with the south of Europe: an exploration with two countries. **Pediatr Obes** 2015; 11(5):403-10. (IF = 3.7).

[11] Martinez-Tellez B, Sanchez-Delgado G, Cadenas-Sanchez C, Mora-Gonzalez J, Martín-Matillas M, Löf M, et al. Health-related physical fitness is associated with total and central body fat in preschool children aged 3 to 5 years. **Pediatr Obes** 2015; 11(6):468-474. (IF = 3.7).

[12] Sanchez-delgado G, Martinez-Tellez B, Ruiz JR. Does chronic aerobic exercise reduce brown adipose tissue activity ? Comment on: Low brown adipose tissue activity in endurance trained compared to lean sedentary men (Int J Obes, 2015). **Clin Nutr** 2015; 2014–5. (IF = 4.5).

[13] Cadenas-Sanchez C, Martinez-Tellez B, Sanchez-Delgado G, Mora-Gonzalez J, Castro-Piñero J, Löf M, Ruiz JR OF. Assessing physical fitness in preschool children: Feasibility, reliability and practical recommendations for the PREFIT battery. **J Sci Med Sport** 2016; 11:910–5. (IF = 3.8).

[14] Sanchez-Delgado G, Martinez-Tellez B, Gil A. Is Brown Adipose Tissue-Mediated Adaptive Thermogenesis the Missing Component of the Constrained Total Energy Expenditure Model? **Ann Nutr Metab** 2016; 69(1):51-3. (IF = 2.5).

[15] Martinez-Tellez B, Sanchez-Delgado G, Boon MR, Rensen PCN, Ruiz JR. Activation and quantification of human brown adipose tissue: methodological considerations for between studies comparisons: Comment on: Hot heads & cool bodies: The conundrums of human BAT activity research**. Eur J Intern Med** 2017; 40:e19-e21. (IF = 2.9).

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Acknowledgements/ Agradecimientos

Al comienzo del doctorado, recuerdo que le preguntaron a Jonatan "-¿Estos chicos tienen veneno?" A lo que dijo: "-Parece que sí, pero veremos a ver". En esos momentos no entendí esa expresión así que hice una búsqueda por mi cuenta. En resumen encontré que en el siglo II D.C. los romanos inventaron una serie de pócimas cuya finalidad era hacerse amar o despertar el deseo sexual en otras personas las cuales llamaron *venenum*. Estas pócimas de origen poco desconocido en algunas ocasiones, intoxicaban y mataban a algunas de las personas que las tomaban. Con el paso de los siglos, el término *venenum* se extendió a todas las drogas, pociones y medicamentos hasta que hoy en día dicha palabra se conoce como **veneno**. La RAE atribuye a dicha palabra las siguientes acepciones: 1. Sustancia que produce en el organismo graves trastornos o la muerte; 2. Lo que es nocivo para la salud; 3. Lo que produce daño moral.

Después de realizar esa búsqueda, leí por primera vez el proyecto FIS que a Jonatan le acababan de conceder: el proyecto ACTIBATE. Tras esa lectura sentí que la curiosidad que despertó en mi dicha temática era similar a lo que una droga, poción o medicamento puede hacer en el organismo. A partir de ese momento la ciencia y esta temática me envenenó con su sistema ordenado de conocimientos. A la misma vez apareció Guillermo, lo más parecido a una novia que he tenido en estos años. Hemos compartido este envenenamiento de una forma que nos hemos retroalimentado pienso que de forma positiva. **Guille (el cabeza)**, eres la persona más talentosa que he visto en mucho tiempo, el nivel de maduración que has alcanzado en tu etapa predoctoral es digno de ser estudiado. Todas las discusiones (buenas y malas) que hemos tenido me han hecho mejorar como persona y como científico. Lo que he aprendido de ti es incuantificable, sé que si no hubiese coincidido contigo en esta dimensión espacio-temporal a día de hoy seguro que sería peor científico de lo que soy hoy en día y probablemente "peor" persona. Todavía recuerdo las veces que me regañabas porque aparcaba ocupando dos plazas en el mercadona o el descubrimiento que me hiciste con el programa Cuando ya no esté. Sé que eres la persona más venenosa de mi entorno y créeme que en esta siguiente etapa voy a echar de menos eso, mucho. Tan solo espero, sueño y deseo no dejar de trabajar contigo y poder en futuro estar trabajando bajo el mismo techo en Granada, sino siempre nos queda la opción de abrir un negocio de schawarmas en el campo del príncipe. Tu curiosidad traspasa límites, te cuestionas todo y así es como se hacen los descubrimientos. Gracias a ti aprendí que no existen límites. Eternamente agradecido a ti.

¡Equipo **ACTIBATE**! Gracias a todos y cada uno de vosotr@s. Esta tesis ha sido posible gracias a vuestr@s sacrificios, (trabajar innumerables fines de semana, puentes, domingos de ramos, navidades, etc). No ha sido nada fácil pero hemos llegado al final y ahora estamos empezando a recoger los frutos. Sois el ejemplo perfecto de lo que Ángela Duckworth definió como Grit (i.e. trabajo y perseverancia como claves del éxito). Con muchos de vosotr@s hemos compartido ese veneno que es el ACTIBATE, veneno del weno... debates científicos hasta altas horas de la

madrugada, estar de botellón y difícilmente dejar de hablar de lo mismo hasta que nos hemos obligado (algunas veces) a parar de hablar de ese tema.

Victoria (la rubia): gracias por tu apoyo y ofrecimiento. Gracias por el sacrificio que has hecho para poder ser parte de este trabajo. Espero que esta experiencia de aprendizaje haya sido muy positiva para ti. Gracias por la infinidad de conversaciones, por entenderme y cuidarme. No olvides nunca de sonreír.

Wendy (la mexicana): todavía tengo resaca de la celebración de tu beca Conociiiiiiiu en Utrecht. Gracias por involucrarte de la manera que lo has hecho en todo este proceso. Apostaste por este proyecto cuando muy poca gente lo hacía y lo hiciste dejando atrás a tu familia y eso es de agradecimiento eterno. Lucha y trabaja para la siguiente fase, no la olvides.

Juanma (el piercings): recuerdo cuando nos conocimos en el máster y decidiste meterte en esta locura de vida. Gracias por estar atento a los detalles y en cuidarnos, aunque nuestras relaciones han tenido sus altibajos, finalmente siempre florece la amistad. Gracias por haber sido el master de las tecnologías y sobre todo de los cacharros esos que supuestamente miden el gasto!

Amaro (amgarrabo): tu capacidad de trabajo y sacrificio es impresionante. Admiro lo que eres capaz de trabajar, sacrificar y conseguir con el simplemente hecho de la perseverancia, que en tu caso tiene un toque de inteligencia abismal. Vas a conseguir lo que te propongas, desde que te conocí allá por 2009 lo has hecho y ahora no va a ser menos. Te deseo lo mejor y espero poder trabajar contigo en los próximos años. No te olvides de vivir!

Ely (Shu Elhy): eres la dulzura del IMUDS por la mañana y la colilla del suelo por la noche, por eso me encantas! Por fin ha llegado tu momento, por fin tienes la tranquilidad que te mereces durante 2 años. Usa este tiempo para brillar, brilla lo más alto que puedas siempre conservando la humildad que tanto te caracteriza. Vales para ser una científica de referencia, nunca lo olvides.

Acosta (er true cabesa): gracias a ti aprendí que existe una colinealidad positiva entre el tamaño de tu almendra y el corazón que tienes. Eres un ejemplo para mí, usa la oportunidad que tienes de ser funcionario para estudiar, y ser mejor. Tienes un gran talento que con el toque de la capacidad de sacrificio que tienes hace que tu combinación sea bárbara. No olvides de dónde vienes para saber muy bien hacia dónde vas. Tienes todas las herramientas para conseguir tocar el cielo, trabaja para ello

Hui y Lourdes, Lourdes y Hui (la china y la amiga, la amiga y la china): disfrutad esta etapa para aprender y disfrutar todo lo que podáis. Perdón si a veces he sido demasiado duro o directo con mis opiniones pero os lo digo con todo el cariño para que mejoremos todos. Hui, para los amigos de verdad (Wii Nintendo o Huawei), eres capaz de trabajar como una china y aún no sé porque xD. Tienes unas virtudes y un potencial del que tú misma no eres consciente, trabaja para descubrirlo ya que puedes deslumbrar el mundo. Lourdes (la penas), gracias por decirle a tu madre que tenías la misma suerte que yo para conseguir tu ansiada beca, pero por suerte no ha sido así. Tengo envidia sana por la oportunidad que tienes. Cuestiónate todo lo que te rodea, se curiosa y trabaja

ladrillo a ladrillo para conseguir vuestro sueño. Podéis llegar a ser las más mejores "mierdas" del mundo ☺.

Lucas (er bigotillos): tienes un corazón del tamaño de tu glúteo. Has tenido momentos complejos durante estos últimos años/meses, pero al final vuelves a tu cauce que es con nosotros. Aprovecha la oportunidad de trabajar aquí con toda esta gente, aprovecha a Jonatan y marca un antes y después en este grupo, porque tienes la capacidad para ello.

MJ (la pucha destilada): probablemente defenderás la tesis antes que las imágenes de composición corporal del cuello. Es broma! O no... Gracias por venirnos a sorprendernos con tus flamantes cambios de composición corporal y la capacidad para beber vino. Mil gracias por la excursión de Sevilla a la Virgen de la Macarena. Tienes una capacidad de trabajo brutal al igual que tu pasión sobre la nutrición deportiva, puedes llegar muy lejos.

Pascual (el lechero por el día, el marrano buscando trufas por la noche): Vas a ser capaz de poner las mejores tetas de toda Alemania. Esfuerzate por conseguir tu sueño sin mirar quien está a tu lado. Eres un ejemplo para mí en muchos aspectos, sobre todo en la capacidad de pintarte la ceja.

Javi (Spiderman): el mejor guitarrista a 4 cuerdas que he conocido nunca! Gracias por ayudarme a mejorar, por ser un gran incentivo venenoso, gracias por recordarme lo que era la ansiedad y gracias por enseñarme tanto. Tu inteligencia supera límites, utilízala para el bien común.

Andrea (la niña...sevillana): gracias por elegir unirte a esta familia de locos, sé que a veces nos has mirado con cara de estos niños están malitos y no es para menos. Disfruta la oportunidad que tienes delante de ti porque es única.

Lucas (el calza participantes): has sido uno de los descubrimientos de este proyecto. Gracias por tu profesionalidad y visión de la cosas. Tienes una capacidad increíble para saber de qué pierna cojea cada persona. Tengo un recuerdo precioso contigo caminando juntos, gracias por ese abrazo que necesitaba en dicho momento. Mucha suerte en tu nueva aventura.

Carmen (del árabe karm): Gracias por tu ayuda corrigiéndonos nuestros textos y pronunciaciones de Spanglish. Mucha suerte con tu tesis. You are the best teacher ever!

Además del grupo humano del ACTIBATE, esta tesis me ha demostrado que el **personal sanitario** que hay en nuestra Junta de Andalucía es increíble y que hoy en día estamos en las mejores manos posibles.

Jose Manuel Llamas-Elvira (el abuelazo): Jose Manuel, tu capacidad humana supera a tu inteligencia cosa que es imposible. GRACIAS Y GRACIAS por sacrificar muchos domingos (n=16) para poder realizar las pruebas del proyecto ACTIBATE, inclusive por amenizarlos con todas las batallas que conoces y tu capacidad de avergonzar a la gente. Tu alegría nos ha empujado a trabajar más duro aún si cabe y este proyecto sin lugar a dudas es mérito tuyo y de Yolanda. Jamás podremos devolverte todo lo que has hecho por nosotros. Es increíble que a unos años de retirarte seas capaz de hacer el sacrificio que has hecho. Nos ha dado una lección a los más jóvenes, siendo un espejo en el que nos podemos y debemos mirar.

Yolanda (la jefa): la palabra admiración se queda corta para describir lo que siento por ti. Eres la madre coraje por referencia, estás dotada de una inteligencia intelectual y emocional bárbara. No sé cómo has podido aguantarnos y como has podido ayudarnos tanto. Tus hazañas son dignas de ser narradas para la posteridad, es increíble que hayas podido estar sacando sangre a 2/3 personas a la vez. A parte de todo esto, tienes una capacidad humana increíble, tus hij@s seguro que están super orgullosos de ti, al igual que lo estamos nosotros. Ya has conseguido tu primer hito académico, pero lo mejor está aún por llegar

No puedo olvidarme de agredecer a todo el personal del Virgen de las Nieves que ha estado trabajando con nosotros y ayudándonos en conseguir este proyecto. MIL GRACIAS.

Esther (la cirujana que fuma): gracias por tu predisposición y sacrificio para poder hacer las biopsias del estudio. Eras capaz de venir de una rotación sin apenas dormir y sacar una sonrisa para ponerte a trabajar. GRACIAS por el trabajo que has hecho es impagable intentaré hacer lo posible para ayudarte a conseguir tu sueño.

Pepe (dr. Nick): Se nota que eres "de Jaén ni pollas" a la legua. Gracias por tu ayuda y trabajo, gracias por las de risas y anécdotas. Jamás olvidaré como me enseñaste a afrontar los desmayos de la gente con la famosa frase de "ya está haciendo el Stephen Hopkins". Gracias y sabes dónde encontrarme para lo que necesites.

También este proyecto nos ha obligado a trabajar en el centro de instrumentaciones biomédicas (**CIBM**):

Josune (la jefa de la jeringa): Gracias por el aprendizaje del orden y la cautela, aunque a día de hoy no soy capaz de aplicarlo ;) Espero que te esté yendo todo genial en UK.

Julio y Fran (Zipi y Zape): no dudasteis en acogernos dentro de la familia CIBM, espero poder devolveros pronto la invitación a gambas o el palco.

Una de las partes más importantes de esta historia y que nunca nos podremos olvidar son nuestros queridos practicums, TFGs y TFMs que han pasado por este proyecto, gracias por vuestra ayuda. Sin embargo MUCHAS GRACIAS a todos los participantes que de forma desinteresada decidieron colaborar en un programa de entrenamiento de 6 meses a cambio de una cantidad de prueba que recibisteis bárbara. Vuestra contribución a la ciencia es más grande que la de cualquier gobierno o fundación privada. Sin vosotros esta tesis no hubiese sido posible ¡GRACIAS!
Paralelamente a este proyecto nos ha tocado compartir espacios y convivencias con PROFITH y Gestafit

Fran Ortega (er payicor): gracias por apostar por nosotros y por mí. Gracias por escucharnos y perdón si alguna vez he dicho/hecho algo que te haya podido molestar. Todavía recuerdo todos y cada una de las llamadas que me has hecho tras cada rechazo predoctoral. Tu apoyo ha sido muy importante en este proceso, gracias por empujarme hacia delante y seguir.

Palma (la clave): básicamente mi primera experiencia académica fue contigo, gracias por ayudarme a seguir en los momentos más difíciles y por formarme durante mis prácticas

docentes, gracias por confiar y en ayudarnos a ser diferentes.

Miguelón (la vin que largo): eres un ejemplo de que hacer si la vida te da limones, ¡Has hecho la mejor limonada de la historia ☺! Gracias por tu apoyo, solidaridad y confianza en nosotros, espero volver a cruzarme contigo pronto.

Ire (canoas): ejemplo en muchos aspectos! Tu capacidad de narcolepsia, contestar emails a horas extrañas, volcar canoa y luego trabajar como una condená hacen que seas una persona a la que no se le puede no querer. Gracias por tu apoyo.

Pepe (my Justin Bieber): He crecido contigo tanto académica como personalmente. Jamás podré olvidar las prácticas que hicimos y la sensación que sentí en aquellos momentos de felicidad plena. Gracias por escucharme y darme los mejores consejos siempre. Tus risas y detalles hacen que seas una de las mejores personas que conozco. Tu inteligencia, sobre todo emocional, traspasas límites. La única espina que tengo contigo es la de no haber trabajado más codo con codo.

Cristi (cofita): gracias por liderarnos en el proyecto de los cofitos. Has sido un ejemplo en muchos aspectos, gracias por aguantarnos y ser la balanza y el toque de cordura que hacía falta en ese equipo de evaluadores.

Jairo (Edward Cullen): gracias por ser un ejemplo de lo que es el trabajo y sacrificio. Gracias por la visita en Holanda y los miles de detalles que has tenido hacia mí. Vas a llegar hasta donde tú quieras.

María (Rodríguez): eres más inteligente y capaz de lo que eres consciente, aprovéchalo. Gracias a ti aprendí la diferencia entre embrismo y feminismo, me has aportado muchísimo como persona. Gracias por enseñarme tanto.

Lidia (α): Gracias por tus detalles y palabras. Eres un ejemplo de persona coraje, eres referente para muchas personas y como no iba a ser menos para mí también. No tienes techo.

Irene (Col de Bruselas): tus clases de patinaje me han ser menos paquete sobre la pista. Gracias por tu involucración y apoyo durante esta fase sobre todo en la recta final, echo de menos esos cafés. Te vas a comer Canada!

Mireia (la de Castellón) y Alex (squirtel): vinisteis de estancia a Granada y os tocó sentaros en la peor mesa del IMUDS, pero gracias a eso aprendí muchísimos de vosotros. Sois simplemente geniales, vuestra humildad e inteligencia no tiene límites, gracias por enseñarme tanto.

Fer (Chewbacca): Nos unió un litrona en Exernet 2014 y gracias a ese momento descubrí a un verdadero amigo. Gracias por tus palabras, consejos y anécdotas que me has hecho vivir. Eres referente, espero ver hasta donde llegas pero predigo que muy alto.

Isaac (Yoda): Has sido uno de los pocos profesores que me han marcado durante la carrera. Gracias a ti he aprendido que hay que intentar hacer las cosas diferentes, que hay que esforzarse a mirar las cosas de otro punto de vista, que hay que salir de la zona de confort. Mil gracias por todos los valores que me inculcaste, gracias a ti hoy soy un investigador con un toque diferente. Me ha encantado que me hayas involucrado en tus locuras y espero poder seguir haciéndolo. Gracias por ser diferente y por demostrar todo lo

que has demostrado durante este tiempo.

Gracias a **Sonsoles**, **Manu Herrador**, **Alberto Soriano**, **Pablo Molina**, **Abel**, **Lucía**, **David Pavón** y **Alex de la O** por vuestro apoyo incondicional desde el IMUDS. Al igual que a mi otra familia predoctoral que "vive" al otro lado del muro (**en la facultad**), muchas gracias por vuestras conversaciones, apoyo y cariño, en especial a mis compañeros de fatigas **Javi Fernández y Artacho**. I would like also to thanks to all the people of the LUMC especially to **Kimberly, Laura, Eline, Maike** and **Milena,** looking forward to seeing you!

Patrick: Officially, your name does not appear as co-supervisor but you have done this role with me since the very beginning; this is why I have placed your name here. Thank you for supporting me all this time, thank you for teaching me how to change my English writing and improve my future proposals. Thanks for the volley-beach summers and the long discussions about life, science and future in the car in our way to Scheveningen. Looking forward to developing new ideas!

Mariëtte: I wrote you an email by first time in December of 2015, and today you are my co-supervisor. It is crazy!, isn't it? Thanks for all your charming support, for helping me with the manuscripts and grants. You have been a wonderful co-supervisor and I am really excited about what will be the next step. I read the lekker book that you and the people of the lab gave me in 2016 "The UnDutchables" I hope in the coming months to starting complain about the delays of 2 minutes of the train for being a real Dutch person.

Ángel: simplemente siento admiración infinita hacia ti. Eres el ejemplo de un científico excelente en combinación con una humilde persona. Gracias por sacar tiempo para nuestros Cafés Conciencia, poder optar a esas clases privadas contigo me ha hecho aprender infinidad de conceptos y formas de trabajar. Gracias y perdón por esas entrevistas que te hemos hecho en algunos viajes. A veces esas preguntas eran quizás demasiado personales como cuáles son tus creencias religiosas, cuya respuesta mantendré en el secreto.

Jonatan: Finalmente hemos conseguido llegar al primer checkpoint, camino duro y con unas cuantas piedrecitas eh? Como Coldplay dijo una vez 'Nobody said it was easy' y ya ves si no fue fácil... Cualquier conjunto de palabras que escriba en este texto son insuficientes para agradecerte lo que has hecho por mí. Gracias a ti conseguí la beca predoctoral más difícil del mundo y fue la de tu apoyo tanto emocional, científico como económico para que desarrollase una carrera investigadora en este país que tanto amamos pero que tan difícil nos lo pone, España. Tú apostaste por mi cuando nadie lo hacía (ni yo mismo). Tú has estado ahí cada vez que he recibido un rechazo de una beca predoctoral (1 FIS, 1 DANONE, 1 MAPFRE, 2 Sabadell, 1 Oriol Urquijo, 3 FPU y el contrato de técnico). Por cada rechazo, recibía una llamada tuya animándome a seguir, a luchar, a trabajar para ser mejor. Básicamente aprendí lo que es la resiliencia. Créeme que todo esto jamás lo olvidaré. Gracias por hacer que nuestra relación esté basada en la confianza mutua. Tengo que reconocer que al principio no era fácil sentarme delante de "Jonatan R. Ruiz" y decirle todo en lo que pensaba diferente. Sin embargo, después del

ACTIBATE hemos conseguido llegar a un maravilloso equilibrio. También querría aprovechar para pedirte perdón. Perdón por haberte quitado tiempo de estar con tu magnifica mujer e hijas, perdón por ser tan chapas con algunos temas. Perdón por tener que aguantarnos tanto a Guille como a mí. Hoy te reconozco que más de una vez nos hemos amotinado y planteado estrategias para convencerte sobre algunas cosas ;) Sé que esto no ha sido tarea fácil y soy consciente de que cualquier otro IP nos hubiese mandado a freir espárragos. Gracias por esa libertad profesional e intelectual, gracias por dejarnos interpretar el "easy chavales, easy", por el "**y si** medimos esta variable?!, **y si** medimos esta otra?!" Esta etapa ha sido mágica gracias a ti. Eres el mentor perfecto, el que cualquier estudiante de doctorado desearía tener y siempre me he sentido muy muy afortunado por darme esta oportunidad. Una vez nos dijiste "Si el alumno no supera al maestro, ni es bueno el alumno ni el maestro", en este caso tengo la sensación de que el alumno no ha sido capaz de superar al maestro, ya que el maestro es muy grande. Siempre has cumplido con tu palabra, ahora espero que puedas cumplirla también. Hoy en día mi futuro tiene más interrogantes que otra cosa, pero sueño con que puedas cumplir tu palabra de traernos de vuelta a la preciosa Granada para poder desarrollar un laboratorio pionero y revolucionario en el mundo del metabolismo energético. Mientras tú nos buscas esa oportunidad, ten por seguro que desde fuera estaremos haciendo lo posible e imposible para que cuando llegue esa oportunidad poder cogerla con la firmeza y experiencia suficiente. ¡MIL GRACIAS JEFE!

Esta tesis ha sido posible a mi **antídoto** que no es más que mi familia y amigos, sin ellos probablemente hubiese perdido la cabeza más aún si cabe en estos 4 y 5 años. Cuando entras en un ambiente tan venenoso como ha sido esta experiencia de aprendizaje, de vez en cuando la mente necesita escapar y que mejor forma que huyendo **a casa**.

Papá y Mamá: mil gracias por el apoyo que me habéis dado durante la carrera y estos años de doctorado sois un ejemplo para mí y no podría haber sido más afortunado de haber nacido bajo vuestro techo. Mil gracias por recibirme en mis estancias en el pueblo y por todos y cada uno de los valores que tengo. Perdón por todos los cumpleaños y fiestas que me he perdido por estar demasiado centrado en esta fase, no volverá a pasar. Os quiero. **Álvaro**, siempre serás el pequeño de la casa, muy orgulloso de lo que estás consiguiendo espero que consigas todos tus sueños, no olvides de dónde vienes para saber hacia dónde vas. Te quiero. Esta tesis va por vosotros.

Por supuesto no podría olvidarme de mi tito **Santi, Grego** y tita **Ara**, gracias por vuestra compañía y ánimo. Tita estas tartas me han dado la vida. Gracias a mis primos y primas en especial a **Ara** y **Santi**, no tenéis límites. Como no agradecer a mis **abuelas**, vosotras empezasteis todo esto trabajando desde la humildad y sacrificio, poco a poco habéis ido construyendo una familia y gracias a eso, los más jóvenes hemos tenido estas oportunidades. Os quiero!

Como no podría ser menos... gracias a mis señores de la noche, amigos desde la infancia que siempre están ahí para cuando uno los necesita. Simplemente, el sentarnos en la misma mesa en

Navidad o Semana Santa me hace valorar la gran suerte que he tenido con vosotros. Mil gracias **Jarry, Alf, JD, Dami, Kike, Jandro, Ana, Sonia, Eva** y **Maribel.**

David (bombero torero), tú has sido clave en este proceso, has sido el apoyo más grande y fuerte que he tenido en Granada. Se que eres un tío de verdad por eso sé que voy a ver como consigues tu sueño. Lucha, trabaja y aguanta, todo tiene su tiempo y finalmente tendrás el éxito que te mereces.

MIL GRACIAS POR DARME ESTA OPORTUNIDAD. *THANK YOU SO MUCH FOR THIS OPPORTUNITY*

Borja

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