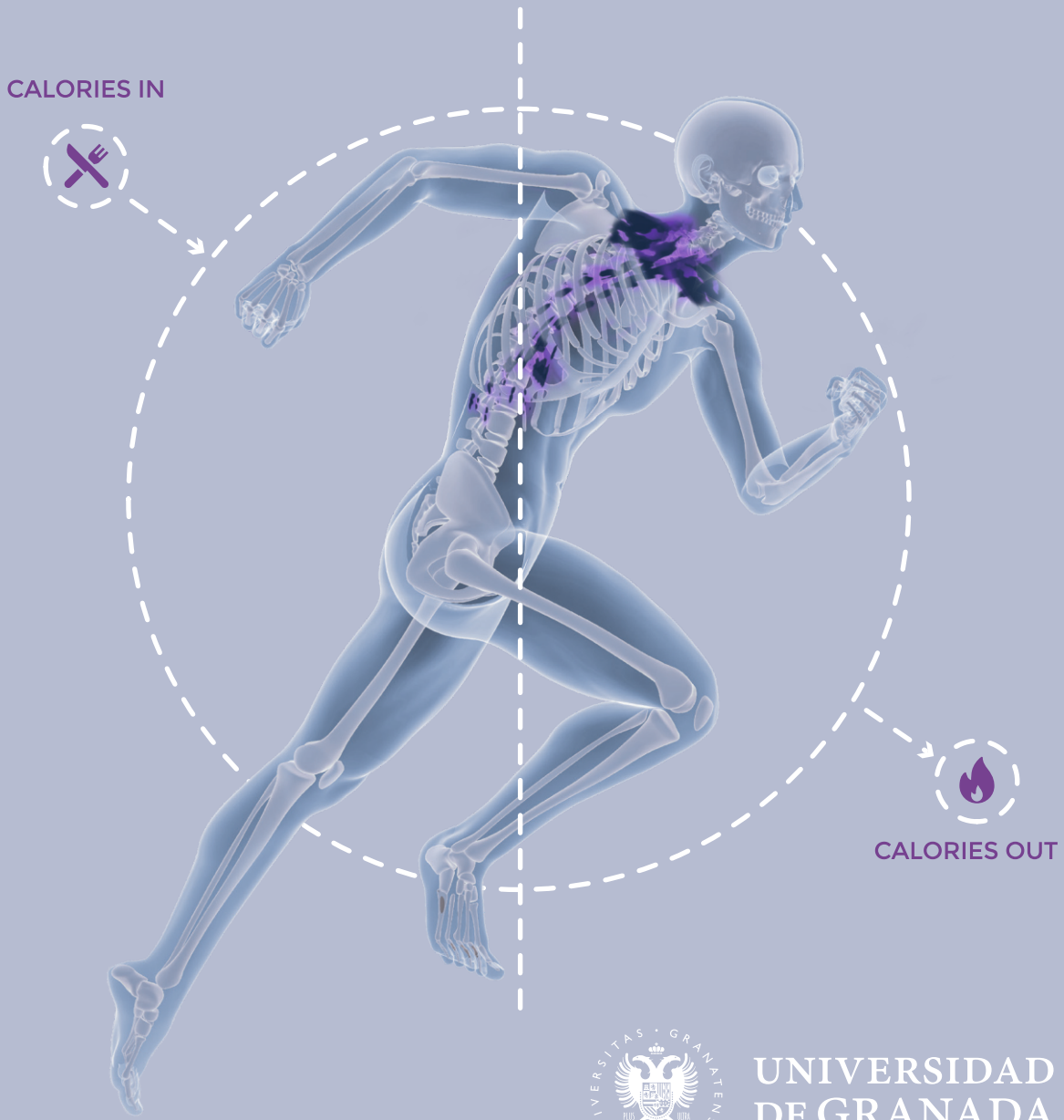
PROGRAMA DE DOCTORADO EN BIOMEDICINA

BROWN ADIPOSE TISSUE AND EXERCISE: IMPLICATIONS ON HUMAN ENERGY BALANCE AND METABOLISM. THE ACTIBATE STUDY

Guillermo Sánchez Delgado



UNIVERSIDAD DE GRANADA

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Tejido adiposo pardo y ejercicio: implicaciones en el balance y metabolismo energético. Estudio ACTIBATE

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Guillermo Sánchez Delgado

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Guillermo Sánchez Delgado

Directores de la Tesis Doctoral [Doctoral Thesis Supervisors]

Jonatan Ruiz Ruiz
PhD
Prof. Titular de Universidad
Universidad de Granada

Ángel Gil Hernández
PhD
Catedrático de Universidad
Universidad de Granada

José Manuel Llamas Elvira
MD, PhD
Jefe de Servicio
Hospital Universitario Virgen de las Nieves

Miembros del Tribunal [Doctoral Thesis Comittee]

Jesús Francisco Rodríguez Huertas
PhD
Catedrático de Universidad
Universidad de Granada, España

Mariana Fátima Fernández Cabrera
PhD
Profesora Titular de Universidad
Universidad de Granada, España

José Antonio López Calbet
MD, PhD
Catedrático de Universidad
Universidad de Las Palmas de Gran Canaria, España

Kirsi A. Virtanen
MD, PhD
Professor
Turku University Hospital, Finland

Denis P. Blondin
PhD
Adjunct Professor
Université de Sherbrooke, Canada

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A mi mejor amiga, a mi mejor maestra,
a quién me dio la vida y me enseñó a vivirla,
a mi madre

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ABSTRACT

In 2009, brown adipose tissue (BAT) was discovered to be present and active in human adults. Since then, BAT has been regarded as a promising target for therapies against obesity and related diseases. However, whether BAT is able to significantly influence human energy balance and metabolism regulation is still unclear. Moreover, there is biological base to hypothesize that BAT activation and/or recruitment takes place in response to physical exercise, possibly being one of the still unknown mechanisms by which exercise exert important beneficial effects on human health.

The main aims of this Doctoral Thesis are to study the role of human BAT in energy balance regulation (section 1), the relationship between BAT and body composition (section 2), the role of BAT in the development of the metabolic syndrome in overweight or obese patients (section 3) and the effect of exercise on BAT volume and activity (section 4). This Doctoral Thesis includes a total of 10 studies.

The results of present Doctoral Thesis show that human BAT volume and activity are not associated with basal, meal or cold-induced energy expenditure and nutrient oxidation rates, neither with energy intake or appetite regulation. BAT volume and activity are not associated either with bone mineral density. On the other hand, BAT volume and fat content, but not its activity, are positively associated with whole-body and central adiposity, while BAT fat content is positively associated with body adiposity. Moreover, metabolically healthy overweight or obese (MHOO) young adults present higher BAT volume and activity, whole-body adaptive thermogenesis and metabolic flexibility than their unhealthy counterparts. Finally, a 6 months exercise training program does not modify BAT volume and activity.

Collectively, the results of this Doctoral Thesis indicate that BAT is not significantly implicated in human energy balance regulation, although BAT volume and fat content are higher in overweight or obese patients, suggesting that BAT hypertrophy and hyperplasia take place in response to body weight increases. Moreover, the higher BAT volume and activity in MHOO participants suggests that BAT expandability could be playing a role on the prevention of the metabolic syndrome. Finally, exercise training seems not to modify BAT volume or activity, although future studies are needed to confirm these findings.

RESUMEN

En 2009 se descubrió que el tejido adiposo pardo (TAP) se encuentra en el adulto humano y es activo. Desde entonces, el TAP se ha considerado una prometedora diana para terapias contra la obesidad y patologías relacionadas. Sin embargo, aún se desconoce si el TAP es capaz de influir de forma significativa en la regulación del balance y metabolismo energético humano. Por otro lado, existe base biológica suficiente para hipotetizar que el TAP se incrementa y/o activa en respuesta al ejercicio físico, siendo éste posiblemente uno de los mecanismos aún desconocidos por los que el ejercicio produce importantes beneficios en la salud humana.

Los objetivos principales de esta Tesis Doctoral son estudiar, el papel del TAP humano en la regulación del balance energético (sección 1), la relación ente TAP y composición corporal (sección 2), el papel del TAP en el desarrollo del síndrome metabólico en pacientes con sobrepeso u obesidad (sección 3), y el efecto del ejercicio sobre el volumen y actividad del TAP (sección 4). Esta Tesis Doctoral incluye un total de 10 estudios.

Los resultados de la presente Tesis Doctoral muestran que el volumen y la actividad del TAP humano no se asocian con el gasto energético y la oxidación de nutrientes basal, en respuesta a una comida o exposición a frío, ni con la ingesta energética o la regulación del apetito. El volumen y actividad del TAP tampoco se asocian con la densidad mineral ósea. Por otro lado, el volumen y contenido graso del TAP, pero no su actividad, se asocian positivamente con el nivel de adiposidad corporal. Además, adultos jóvenes con sobrepeso u obesidad metabólicamente sanos presentan mayor volumen y actividad del TAP, y mayor termogénesis adaptativa y flexibilidad metabólica general que sus pares metabólicamente enfermos. Finalmente, un programa de entrenamiento físico de 6 meses de duración no modifica el volumen y la actividad del TAP.

En conjunto, los resultados de esta Tesis Doctoral indican que el TAP no está significativamente implicado en la regulación del balance energético humano, aunque el volumen y el contenido graso del TAP son mayores en personas con sobrepeso u obesidad, lo que sugiere que se produce hipertrofia e hiperplasia del TAP en respuesta a incrementos de peso corporal. Además, el mayor volumen y actividad del TAP en participantes con sobrepeso u obesidad metabólicamente sanos sugiere que la expansibilidad del TAP podría jugar un papel en la prevención del síndrome metabólico. Por último, el ejercicio físico parece no modificar el volumen o la actividad del TAP, aunque futuros estudios son necesarios para confirmar estos hallazgos.

ABBREVIATIONS

ACC: Accelerometry.

ACTIBATE: Activating brown adipose tissue through exercise study.

Akt: protein kinase B.

AMPK: AMP-activated protein kinase.

ANOVA: Analysis of variance.

ANCOVA: Analysis of co-variance.

APP: Energy intake and appetite regulation.

ATP: Adenosine triphosphate.

AUC: Area under the curve.

BAT: Brown adipose tissue.

BAIBA: β-aminoisobutyric acid.

BCa: Body composition assessment.

BDNF: Brain-derived neurotrophic factor.

BIC: Basal indirect calorimetry.

BMD: Bone mineral density.

BMI: Body mass index.

BMR: Basal metabolic rate.

cAMP: cyclic adenosine monophosphate.

CCM: CCM Express (Medgraphics Corp, Minnesota, USA).

cGMP: Cyclic guanosine monophosphate.

CHOox: Carbohydrates oxidation.

CI-NUTox: cold-induced nutrient oxidation rates.

CIT: Cold-induced thermogenesis.

Clo: Clothing insulation value.

CT: computerized tomography.

CV: Coefficient of variance.

CVD: Cardiovascular disease risk factors.

DXA: Dual-energy x-ray absorptiometry.

EE: Energy expenditure.

Fatmax: The intensity at which maximal fat oxidation is reached during exercise.

FATox: Fat oxidation.

FGF 21: Fibroblast growth factor 21.

FNDC5: Fibronectin type III domain containing 5.

FOXO1: forkhead box O1 transcription factor.

GDF-8: Growth differentiation factor 8.

GMP: Guanosine monophosphate.

HDL: High-density lipoprotein cholesterol.

HOMA: Homeostatic Model Assessment.

HRres: Heart rate reserve.

HU: Hounsfield Units.

IC: Indirect calorimetry.

IL-4: Interleukin 4.

IL-6: Interleukin 6.

IL-13: Interleukin 13.

LDL: Low-density lipoprotein cholesterol.

MCT-1: Monocarboxylate transporter 1.

MET: metabolic equivalent.

MFO: Maximal fat oxidation during exercise.

MFOa: Maximal fat oxidation assessment.

MGU: Ultima CardiO2 (Medgraphics Corp, Minnesota, USA).

MHO: Metabolically healthy obese.

MHOO: Metabolically healthy overweight or obese.

MIT: Meal-induced thermogenesis.

mRNA: Messenger RNA.

MUO: Metabolically unhealthy obese.

MUOO: Metabolically unhealthy overweight or obese.

NAD: Nicotinamide adenine dinucleotide.

NST: Non-shivering thermogenesis.

PA: Physical activity.

PAL: Physical activity level.

PGC-1α: Peroxisome proliferator-activated receptor γ coactivator 1α.

PET: Positron emission tomography.

PET-CT: Positron emission tomography and computerized tomography.

POMC: Pro-opiomelanocortin.

PPARγ: Peroxisome proliferator-activated receptor gamma.

PPIC: Post-prandial indirect calorimetry.

p38MAPK: p38 mitogen-activated protein kinases.

RER: Respiratory exchange ratio.

RM: Repetition maximum.

RMR: Resting metabolic rate.

SD: Standard deviation.

SkTa: Skin temperature assessment.

sLR11: Soluble form of the low-density lipoprotein receptor relative.

SNS: Sympathetic nervous system.

SS: Steady state.

SSt: Steady state time.

STAT3: signal transducer and activator of transcription 3.

SUV: Standardized uptake value.

SUVBM: Standardized uptake value expressed as a function of body mass.

SUVLBM: Standardized uptake value expressed as a function of lean body mass.

TEE: Total energy expenditure.

Texpo: Outdoor and personal environmental temperature.

TGF-β: Transforming growth factor beta.

TI: Time interval.

UCP-1: Uncoupling protein 1.

VAS: Visual analogue scales.

VCO2: Whole-body carbon dioxide production.

VE: Minute ventilation.

VO2: Whole-body oxygen consumption.

VO2: Maximum oxygen consumption during exercise.

WAT: White adipose tissue.

5min-SS-2P: The most stable 5-minute period of every half part of the cold exposure.

5min-SS-4P: The most stable 5-minute period of every forth part of the cold exposure.

5min-TI: mean values of every consecutive 5-minute period.

12,13-diHOME: 12,13-dihydroxy-9Z-octadecenoic acid.

^{18}F-FDG: ^{18}F-Fluorodeoxyglucose.

GENERAL INTRODUCTION

OBESITY: AN UNSOLVED PROBLEM OF ENERGY BALANCE REGULATION

Life expectancy has continuously increased during last decades thanks to advances in medicine and economic development. Consequently, age-related non-communicable chronic diseases are the main public health burden in developed and developing countries. Among them, obesity is considered to be a pandemic that has increased exponentially during the last decades. Currently, worldwide overweight and obesity prevalence is estimated to be as high as 36.9% an 38.0% in men and women respectively [1]. Prevalence is even higher in developed countries [1]. Data from the European Health interview surveys (EU, Eurostat) indicate that more than half of the EU population is overweight or obese. It was estimated that in 2010, obesity caused 3.4 million of deaths worldwide [1]. Obesity is an important risk factor for the development of chronic diseases such as cardiovascular disease, type II diabetes mellitus, chronic kidney disease and several types of cancer [2].

Cardiovascular disease is the number one cause of death globally. Data from the World Health Organization indicate that an estimated 17.7 million people died from cardiovascular disease in 2015, representing 31% of all global deaths. Metabolic diseases are other non-communicable chronic diseases reaching pandemic level. Data from the International Diabetes Federation indicates that 382 million people had diabetes in 2013, and this number is estimated to increase to 592 million by 2035. Diabetes caused 5.1 million deaths in 2013.

The obese phenotype is likely to be the result of complex interactions between the individual's genetic background, environmental, behavioral and socioeconomic factors. However, in simple terms, obesity is the result of chronic excess of energy intake over EE. Therefore, establishing a negative energy balance is a requisite for achieving weight loss (i.e. higher EE than energy intake). However, compensatory mechanisms, both physiological and behavioral, in response to short-term negative energy balance make it very difficult to establish a long-term energy deficit and sustainable weight loss [3]. Thus, currently, there is no non-invasive therapy capable of inducing sustainable weight loss.

Body weight changes (i.e. weight loss or weight gain) are associated with changes (i.e. declines or increases) in EE. Those changes are mainly explained by changes in lean mass, the main metabolically active component of the body [4]. Nonetheless, relevant changes in energy balance take place in response to body weight changes independently of changes in lean mass [5]. This group of changes, so-called metabolic adaptation, is produced in order to maintain the current body weight and composition, which is physiologically sensed as homeostatic [6]. Weight loss induce reductions in the resting EE, in the diet-induced thermogenesis, but also in the activity EE [4,7]. Metabolic adaptation is partly explained by a growing number of physiological processes (e.g. altered efficiency of free fatty acid oxidation in skeletal muscle, cycles consuming ATP without a net change in products, changes in the ATP-costs per muscle contraction, etc.) [4]. Importantly, metabolic adaptation determines the capacity of weight loss in response to an intervention [3], or even the propensity to weight regain [8]. Moreover, changes in

response to modification of body weight affect not only EE, but also energy intake regulation [3,4].

Although much has been done during the last decades, it is evident that our current knowledge about energy balance regulation is insufficient to combat the obesity pandemic and new approaches have to be harnessed and exploited [3]. Among the promising therapeutic targets under study, BAT has been regarded as a promising therapeutic target to tackle the obesity pandemic during the last decade [3,9,10].

BROWN ADIPOSE TISSUE: A NEW HOPE IN THE FIGHT AGAINST OBESITY AND METABOLIC DISEASES

In mammals, adipose tissue is found in two forms: WAT and BAT. These two tissues have opposite roles in whole-body energy metabolism. WAT has the ability to store energy in the form of triacylglycerol and to release energy in the form of free fatty acids and glycerol, whereas BAT is a highly thermogenic tissue whose main function is to produce heat for maintaining body temperature in mammals, by oxidation of glucose and lipids [11]. BAT is characterized by a light pink to dark red tone due to the high vascularization and the cytoplasm, which contains small fat-filled droplets and a large number of mitochondria [11]. High vascularization is necessary for nutrients and oxygen supply and for heat dissipation, and for the mitochondria to oxidize glucose and triacylglycerols [11]. The stored triacylglycerol depots are necessary for fast energy supply, and the SNS innervation is needed for fast activation of the tissue [12]. For maintenance of prolonged thermogenesis, the tissue receives substrates (fatty acids and glucose) from the circulation. Ultimately, heat production takes place through the uncoupling process, which is mediated by UCP-1, a unique inner-membrane mitochondrial protein for BAT [11].

In humans it was for long believed that BAT was only present in newborns and was responsible for NST [13], yet, it was thought to be irrelevant in adults. However, serendipitously, radiologists using the radiotracer ^{18}F-FDG in PET-CT to detect metabolically active tumors [14,15] found competing areas with high rates of glucose uptake that were symmetrical in nature [16]. These areas were most commonly localized in the supraclavicular and neck regions [17]. The presence of active BAT in adult humans and its metabolic significance for human physiology was firstly claimed in 2007 [17] and finally recognized in 2009 [18–22]. Currently, there is no doubt that this unique tissue exists and is thermogenically active in human adults. BAT activity seems to decrease with age [20,23–25], be inversely correlated with BMI [20,23] and visceral adiposity [22,25], and be lower in men than in women [15,20,23], but the sex differences has not been confirmed in other studies [26]. Consequently, it has been regarded as a promising therapeutic target for obesity and related comorbidities [9,10,27,28].

It is important to note however that the observed associations of BAT with BMI and sex should be interpreted cautiously when an individualized cooling protocol has not been carried out prior to the PET-CT scan [29]. BAT main function is to contribute to NST, and thus it is supposed to be fully activated when mammals are exposed to a temperature near the shivering threshold (i.e. the temperature when shivering starts). Lower temperatures are needed to induce shivering in males compared to females and

in obese compared to lean individuals [29]. Thus, the higher BAT activity obtained in females and in lean individuals could be biased by indoor temperatures, which may be low enough to induce a relevant NST in females and in lean individuals but not in males or obese individuals. Future studies should assess BAT activity and mass after an individualized cooling protocol [29,30]. Moreover, the SUV threshold, considered as the minimal radiotracer uptake to quantify a voxel as BAT, should be expressed relative to lean body mass [31,32], which was not done in most humans studies showing an inverse association between BAT and BMI or whole-body adiposity. This methodological issue could have biased the relation between BAT and BMI or whole-body adiposity, and further studies are needed to fully understand this association.

Findings from studies performing PET-CT after a personalized cold exposure has served to elucidate that BAT is highly prevalent in adult humans (near 100%) [19,33]. Moreover, Lee et al. [33] showed that even BAT negative subjects on a PET-TC exploration possess a certain amount of BAT. Recently, another type of cells called brown-in-white, ("Brite") or "beige" cells, in WAT of both rodents and humans have been found [34]. Beige adipocytes possess a multilocular morphology, enriched mitochondria and express the brown adipocyte-specific UCP-1 [34–37]. They are peculiar in that they share characteristics with white and brown adipocytes, and their development is regulated by diverse factors in an endocrine, paracrine and autocrine fashion [38]. The development of these thermogenically competent cells in WAT is greatly enhanced in response to chronic cold exposure or prolonged β-adrenergic stimulation, and the occurrence of these cells is associated with resistance to obesity, type 2 diabetes and other metabolic diseases [39].

ROLE OF HUMAN BROWN ADIPOSE TISSUE IN ENERGY BALANCE

Increasing thermogenesis has been considered one of the options to prevent and combat obesity and related comorbidities. In mice, BAT is responsible for 20% [40] of both BMR and adaptive thermogenesis, i.e. CIT and MIT [11], and can account for up to 60% of total EE when fully stimulated [41,42]. In humans, since its re-discovery, BAT has emerged as a promising target for increasing human EE [9,43–45]. However, human BAT physiology is still in its infancy. First studies have tried to answer two main questions related to its role in human energy balance: i) Is human BAT active during resting conditions in a thermoneutral environment, and after a meal? ii) Is human BAT thermogenic capacity sufficient to significantly impact on EE and body weight management?

Human BAT becomes metabolically active upon cold exposure in most, if not all, individuals [19,46–53]. Cold exposure, even to mildly lower temperatures, significantly increases oxidative metabolism [46,52,53], blood flow [46,53], glucose uptake [19,47–50,54], and fatty acid uptake [52,55] in human BAT. Noteworthy, the main function of BAT is to produce heat in order to maintain the core body temperature when mammals are exposed to cold [11]. Consequently, it seems plausible that, although it can be recruited upon cold exposure, human BAT is not metabolically active in thermoneutral conditions. In fact, initial studies assessing BAT in thermoneutral environments by ^{18}F-FDG PET-CT in adult humans showed that only 3-20% of people presented detectable BAT [14,15,20,56,57], suggesting that in most individuals BAT hardly takes up glucose in thermoneutral conditions. Later, two

studies using $^{15}O[O_2]$ for PET-CT scans for BAT assessment showed that BAT is indeed metabolically active in thermoneutral conditions to a similar extent than other tissues such as WAT, and that this activity corresponds to approx. a half of the metabolic activity after maximal cold-activation, which definitely proves that human BAT is thermogenically active in thermoneutral environments [46,53].

Whether BAT activation occurs after a meal ingestion in humans has been mainly studied using ^{18}F-FDG, presenting controversial results. Scholgh et al. [58] reported that BAT ^{18}F-FDG uptake did not increase after an hypercaloric meal, although they found an increased ^{18}F-FDG uptake after cold exposure. Vrieze et al. [59] showed that cold-induced BAT ^{18}F-FDG uptake was reduced after eating, in comparison to fasting state. In contrast, Hibi et al. [60] showed that MIT was higher in participants with detectable BAT ^{18}F-FDG uptake than in participant without detectable BAT ^{18}F-FDG uptake. In line with it, Vosselman et al. [61] found higher BAT ^{18}F-FDG uptake after eating, but they showed no association between MIT and BAT activity, which may suggest that increased ^{18}F-FDG uptake is a consequence of high levels of insulin. Of note, it was shown that insulin induced ^{18}F-FDG uptake does not necessarily means higher thermogenesis, since insulin infusion increases ^{18}F-FDG uptake without increasing BAT blood flow [26]. This is in contrast with cold exposure, which increases both ^{18}F-FDG uptake and BAT blood flow [26]. Interestingly, U Din et al. [62] assessed BAT postprandial metabolism by means of $^{15}O[O_2]$, $^{15}O[H_2O]$, and ^{18}F-FTHA, and compared it with that of cold exposure. They also compared gene expression in human BAT after eating a meal and compared it with WAT gene expression. They elegantly showed that BAT oxygen consumption and blood flow increase after eating a carbohydrate-rich meal to a similar extent than during mild cold exposure. Moreover, they found that despite the BAT non-esterified fatty acids uptake was reduced after eating, this uptake is highly correlated with BAT oxygen uptake and blood flow up-regulation. This, together with an increased expression of several genes involved in fatty acids metabolism suggest a key role of lipid metabolism in BAT thermogenesis, even in conditions in which whole-body metabolism relies mainly on CHOox [62].

BAT activation does not necessarily mean that it significantly contributes to whole-body EE. In fact, a debate about the maximum possible contribution of human BAT to thermogenesis is still ongoing [63]. Assuming rodent bioenergetics data, it has been estimated that 50 g of activated BAT could contribute approx. 5% to resting EE [21]. A 5% chronic increase in resting EE turns to approx. 100 kcal/day, which might translate into a loss of approx. 4.7 kg of fat mass yearly, assuming no compensatory responses to energy EE [64]. Most optimistic estimations, also using rodent bioenergetic data, showed that, if maximally and continuously activated, 250 g of BAT in a young male could account for as much as 520 kcal/day [44]. Based on these estimations, activated BAT might induce weight loss and influence the propensity to become obese, and thus the risk of developing type 2 diabetes and other related metabolic diseases. It should be noted, however, that the BAT blood flow up-regulation by cold has been observed to be greater in mice than in humans, and that BAT glucose uptake capacity in humans is 10 times lower than in rodents [46]. Van der Lans et al. [54] showed an increase in NST after 10 days of cold acclimation (16% of RMR) and concluded that this was attributable to BAT recruitment. They based this assumption on the finding that skeletal muscle mitochondrial uncoupling did not increase in a vastus lateralis muscle biopsy [54]. It should be noted,

however, that deep skeletal muscles seem to be more representative for muscle NST than the more superficially located vastus lateralis [52]. On the other hand, studies using $^{15}O[O_2]$ for assessment of BAT activity by PET-CT showed that cold-activated BAT is only a minor contributor to human EE (i.e. only 1% of the increase of CIT) [46,53,62]. Following these less optimistic figures, the maximum contribution of BAT to EE would be as modest as 15-25 kcal/day [46], or even lower [53]. Moreover, this $^{15}O[O_2]$ approach suggested that NST in humans relies mostly on skeletal muscles [46], as in most large mammal species [3]. This pessimistic perspective on the possibilities of BAT to significantly impact whole-body EE are reinforced by calculations considering reported human BAT blood flow and general bioenergetic data (i.e. normal hemoglobin saturation and oxygen extraction capacity) [65].

Taken together, recent evidence suggests that human BAT may not contribute as much as initially thought to human EE, while NST relies mostly on muscle metabolism, which would implicate that BAT does not directly increase EE sufficiently to contribute to substantial weight loss [46,52,53,65,66]. However, it has been recently proposed that BAT may not directly contribute to NST, but does so in an endocrine manner [53]. This would explain why many studies found a positive association between BAT volume and/or activity and whole-body NST [26,48,53,67]. Skeletal muscle metabolic activity upon cold exposure seems to be muscle group-dependent, being notorious in deep and centrally located muscles, while much less prominent in superficial muscles [52]. Crucially, muscle groups that exhibit high cold-induced ^{18}F-FDG uptake are located close to BAT depots, i.e. longus colli, scalene, and sternocleidomastoid near the BAT supraclavicular and cervical depots; psoas major near the BAT suprarenal depots [52,53,68]. Therefore, it is tempting to speculate that the potent BAT secretome [69] is orchestrating non-shivering and shivering thermogenesis [3] in these muscle groups [70,71]. This intriguing hypothesis [53] needs to be further studied.

Importantly, it should be noted that EE is a potent driver of energy intake [72–75], and therefore, even if BAT significantly contributes to human EE, its possible role could be to increase energy intake rather than creating a negative energy balance through raised EE. These issues suggest that the link between BAT and appetite regulation should be more rigorously examined to fully understand the contribution of BAT to human energy balance [76]. In mice, there are some endocrine mechanisms by which BAT activity significantly influences energy intake regulation [77,78]. Moreover, in human, BAT volume seems to be associated with the basal concentrations of some peptides involved in the appetite regulation system [79]. However, to date, no study has examined the role of BAT in energy intake regulation in humans.

ROLE OF HUMAN BROWN ADIPOSE TISSUE IN METABOLIC HEALTH BEYOND ENERGY BALANCE

Beyond inducing a negative energy balance, BAT activation could also exert beneficial metabolic effects. These effects may derive from the ability of BAT to oxidize glucose and lipids, resulting in euglycemic and hypolipidemic effects [80]. If BAT is able to increase the clearance and oxidation of excess glucose and lipids, it might therefore delay the development of peripheral insulin resistance. Berbeé et al. [81] showed in hyperlipidemic APOE*3-Leiden CETP mice that BAT activation by β3-adrenergic receptor stimulation not only increases EE but also decreases plasma triacylglycerols and cholesterol levels. They

demonstrated that BAT activation enhances the selective uptake of fatty acids from triacylglycerols-rich lipoproteins into BAT, subsequently accelerating the hepatic clearance of the cholesterol-enriched remnants, and ultimately attenuating the atherosclerosis development [81]. In summary, BAT importantly contributes to rodent's homeostasis by functioning as a nutrient sink with lipids and glucose lowering effects [80–83]. Whether this is possible in humans remains to be elucidated [80]. Initial findings in humans showed that cold-activated BAT its associated with improvements in cholesterol metabolism in human patients with hypercholesterolemia, underscoring the potential of BAT activation as a possible anti-atherogenic treatment [83]. Moreover, more studies are needed to better understand if BAT activation is able to favor the restoration of peripheral insulin sensitivity, decrease the amount of insulin needed to maintain euglycemia, and prevent β-cell dysfunction [80]. Interestingly, a recent study showed that temperature-acclimated BAT is able to modulate insulin sensitivity in humans [84].

BAT could also significantly impact human energy homeostasis and health status by functioning as an endocrine organ [69,85]. Several BAT-derived molecules acting in a paracrine or autocrine manner have been identified [69]. Moreover, BAT can release molecules to the bloodstream that act on other tissues and organs [69]. FGF 21, IL-6 and neuregulin 4 are among the first BAT-derived endocrine factors to be identified [69].These endocrine mechanisms are thought to be behind the metabolic benefits of BAT transplantation in rodents. Previous studies using BAT transplantations showed improved glucose homeostasis regardless of insulin, and in parallel with increased plasma levels of leptin, adiponectin, and FGF21 [86–88]. In humans, a 10 day cold-acclimation can significantly improve insulin sensitivity in parallel to BAT activation [68,89], which suggest that the endocrine connection between BAT and skeletal muscle exist in humans.

Whether BAT significantly contributes to human metabolic health either being part of the nutrient clearance system or as a secretory organ still needs to be clarify. There is a subset of obese individuals characterized by a lower risk of obesity-related cardio-metabolic complications [90,91], the so called MHO phenotype. MHO individuals are obese but do not have dyslipidemia, hyperglycemia, type 2 diabetes mellitus, or hypertension [90,91]. MHO individuals have a lower risk of incident type 2 diabetes and cardiovascular diseases [92–96], as well as lower risk of heart failure compared with normal-weight individuals with the metabolic syndrome [97]. Estimates indicate that one in every three individuals with obesity are MHO [98]. Therefore, studying whether the intriguing MHO phenotype is characterized by increased BAT metabolism may be of help on understanding the role of human BAT in metabolic health.

Finally, recent studies have suggested a link between BAT and bone metabolism [99,100], mainly in women [101,102]. Lee et al. [101] reported a positive correlation of BAT volume with total and spine BMD in women, independent of fat and lean body mass, which suggest a possible regulatory link between brown adipogenesis and bone density in humans. This finding concurs with another study that reported lower BMD and BAT mass in women with anorexia nervosa [102]. Interestingly, studies conducted in animal models with defective brown adipogenesis also showed a reduced bone mass [103,104]. Further studies are warranted to elucidated whether BAT activation induces improvement in BMD, and whether BAT can be used as an anti-osteopenia and osteoporosis treatment.

EXERCISE AS A HEALTH PROMOTING STIMULUS: A MYRIAD OF MECHANISMS TO BE DISCOVERED

Increasing the amount of physical exercise effectively improves physical and mental health of persons of any age [105], improves happiness, and makes them more productive [106]. Exercise not only prevents the development of many chronic diseases, but is also an excellent therapeutic intervention for obesity, type 2 diabetes, dementia, Alzheimer, certain types of cancer and many other ailments [105,106]. In terms of efficacy, exercise can be as beneficial as the drugs that are prescribed for many of these diseases, for example for obesity [107] or type 2 diabetes [108,109].

Possibly, the strongest evidence showing the effects of exercise on improved age-related non-communicable chronic diseases comes from the Diabetes Prevention Program Study conducted in the USA, which was stopped early because of efficacy [109]. This placebo-controlled randomized trial demonstrated in 3,234 adults at risk for type 2 diabetes that a life-style intervention involving modest nutritional changes and 150 min per week of continuous, moderate-intensity endurance exercise (that is, brisk walking) for ~3 years is superior to pharmacological intervention (using metformin) and to placebo in preventing the progression of type 2 diabetes in people with impaired glucose control. Interestingly, in a 10 year follow up, the cumulative incidence of type 2 diabetes remained lowest in the exercise group [110]. Improving PA is, unsurprisingly, one of the foremost mandates of the world health organization to attenuate the pandemic nature of obesity and type 2 diabetes [111].

A recent comprehensive review summarized the extensive evidence for the health benefits of regular PA [105]. Pedersen and Saltin presented evidence based support for exercise treatment of 26 different conditions including psychiatric, neurological, metabolic, cardiovascular, and pulmonary diseases, musculoskeletal disorders and cancer [105]. Their overarching conclusion was that “The evidence suggests that in selected cases exercise therapy is just as effective as medical treatment and in special situations more effective or adds to its effect”. They also stressed that “the accumulated knowledge is now so extensive that it has to be implemented.”

Exercise is able to modify body composition, causing a reduction in fat mass and abdominal fat and counteracts loss of muscle mass during dieting [105]. Moreover, physical exercise prevents weight gain, even after a weight loss program [105]. In addition, exercise is able to prevent and reverse insulin resistance, improve lipid profile and improve blood pressure levels [105]. Altogether means that physical exercise is a powerful tool for the prevention and treatment of the metabolic syndrome.

The effects of exercise on some component of total EE, such as MIT and CIT, remain largely unknown [112,113]. Recently, in an outstanding study, Pontzer et al. [7] showed evidence supporting the Constrained TEE model [114], by objectively measuring TEE and PA in free living humans. The Constrained TEE model proposes that TEE increases linearly with PA until it reaches a plateau above which the effect of PA on TEE is negligible. Pontzer et al. [7] also showed that the plateau in the PA-TEE relationship is not explained by a decrease in resting EE [114], but by a decrease in the activity EE component itself. Besides an obvious reduction in non-exercise activity thermogenesis, the magnitude of the reduction of activity EE component points out to an additional reduction in non-muscular EE (e.g.

reproductive activity or somatic maintenance) [7]. Clarifying the mechanism behind the decrease in non-muscular EE is crucial to fully understand the PA-TEE relationship.

Importantly, despite there is mounting evidence that exercise benefits numerous aspects of health, the physiological mechanisms by which such benefits occur are poorly understood [115]. Therefore, given the scientifically proven benefits of exercise on the prevention and treatment of many age-related non-communicable chronic diseases, identifying the physiological mechanism by which the exercise-induced health benefits are exerted would potentially uncover new therapeutic targets and strategies for many age-related non-communicable chronic diseases. Recently, BAT activation and WAT browning have been proposed as a potential part of these unknown mechanisms by which exercise exert beneficial effect on human health [116–119].

ROLE OF EXERCISE IN BROWN ADIPOSE TISSUE METABOLISM

Exercise increases EE and heat production. Since BAT produces heat and consumes energy, it would be expected that BAT is downregulated in response to exercise [120]. However, exercise stimulates several metabolic pathways that regulate BAT and/or browning [116,117,119,121,122]. Besides secretion of potentially BAT-activating catecholamines [123], exercise increases BAT activators that act independently of the stimulation of the SNS, including peptides/hormones [124–132] and metabolites [133,134]. These factors can act on BAT function in an endocrine, paracrine, or even autocrine manner. It was therefore suggested that exercise could be an activator of BAT [116,117,124,135–139].

To date, most empirical evidence regarding the effect of exercise on BAT is derived from animal models. Regarding classical BAT, results are controversial [140]. In 2004, Cannon and Nedergaard [11] suggested that BAT is likely to be hypoactive during exercise, which was indeed confirmed by several studies in animal models [141–145]. Segawa et al. [141] reported no effect on the thermogenic activity of BAT in rats after 9 weeks or running training (5 days/week) [141], and Shibata and Nagasaka [143] reported that daily running for 5 weeks did not change the size of interscapular BAT. Similarly, Wickler et al. [144] showed that running on a treadmill 90 min/day for 6 weeks did not have any effect on resting oxygen consumption, norepinephrine-induced oxygen consumption, and BAT blood flow. In contrast, De Matteis et al. [134] showed an increased parenchymal vascularization of interscapular BAT, which concur with other studies that showed that a 9 week exercise training increases angiogenesis in WAT [146]. They also showed that 1-week running training could be an enough stimulus to induce browning in the visceral fat [134]. These findings concurred with those reported by Slocum et al. [147] that showed that exercising 60 min/day over 7 days up-regulated mitochondrial UCP-1 in the BAT, and that this up-regulation correlated with body weight loss in mice. Recently, Flouris et al. [140], in a systematic review, concluded that exercise is not a relevant stimuli for the expression of UCP1 in classical BAT except when the animals consume a high-fat diet, exercise is combined with cold-exposure, or animals present an endogenous low level of BAT.

Besides the effect of exercise on murine classical BAT, several studies (including different exercise modes, animal phenotypes, and training durations) have consistently

shown an important exercise-induced browning of WAT [119,148,149]. Of note is however that murine results related to thermogenic metabolism are even more difficult to extrapolate to humans than other physiological processes, given that small (e.g. murine) and large (i.e. humans) mammals seems to regulate thermogenesis by different mechanisms [3].

There is a debate about the physiological meaning of exercise-induced beige stimulation and several hypotheses have been proposed: I) Wu et al. [139] found that BAT and beige tissues are antagonistically regulated in response to exercise, with classical BAT being suppressed and beige adipose tissue recruited. Therefore, they proposed that the exercise induced browning could represent a compensatory mechanism for the inhibition of classical BAT. Classical BAT is located nearer the core areas, and thus, is more effective on whole body warming than beige adipose tissue. Therefore, exercise-induced reduction of BAT activity would result in an impaired capacity to generate heat, as expected for an exercise-induce adaption, which is in agreement with the previously observed reduction of CIT and cold-acclimation in response to exercise in rodents [150,151]. However, BAT function is not only to generate heat, but to participate in the energy metabolism regulation (e.g. adjusting the metabolic rate according to energy availability). Therefore, they suggested that the impaired BAT thermoregulatory function as a consequence of exercise might be compensated by beige stimulation, which would exert the same metabolic regulation but a lower heat generating role [139,152]. II) Alternatively, the exercise-induced browning could represent a mechanism involved in the exercise-induced improvement in redox capacity [153,154]. This would be in coherence with the findings of De Matteis et al. [134] who found an increment in PCG-1α expression and an augmented presence of MCT-1 in brown adipocytes membrane, without significant changes on UCP-1 expression, which also concurs with the reduced CIT in response to exercise [151]. III) On the other hand, it has been proposed that low-to-moderate intensity muscle contractions during exercise could mimic muscle shivering and elicit a similar muscle hormone secretion, which ultimately would signal in favor of increasing NST by beige adipocyte recruitment [124]. IV) Moreover, it has been suggested that WAT browning could be an indirect consequence of the exercise-induced reduction in the hypothalamus inflammation, together with the increased insulin and leptin sensitivity. Those hypothalamic changes would lead to an increased activation of POMC neurons, which possibly results in WAT browning [155]. V) Another physiological role of the exercise-induced WAT browning may be an endocrine function of BAT/beige adipocytes during exercise, facilitating muscle function. In this sense, molecules such as myostatin [70] and 12,13-diHOME [71] are secreted by BAT and regulates muscle function. VI) Finally, it also has been suggested that the exercise-induced WAT browning could just represent a compensatory response to the loss of insulation derived from the reduction of fat mass, and white adipocyte size [119].

Importantly, besides the modulation of the catecholamine secretion, there are several endocrine mechanisms that may provide mechanistic explanations for the exercise-induced regulation of BAT and WAT browning (Figure 1):

Sympathetic nervous system

Following the exposure to cold temperatures or acute food intake, the brain coordinates the activation of SNS. In the mature brown adipocytes, released norepinephrine bind to β-adrenergic receptors that are coupled with stimulatory G-proteins that activate adenylate

cyclase, contributing to the activation of cAMP, protein kinase A, and p38MAPK, which subsequently activates lipolysis stimulating enzymes such as hormone-sensitive lipase, adipose triacylglycerol lipase and monoacylglycerol lipase [156]. The resulting increase in free fatty acids activates UCP-1.

Exercise stimulates SNS and catecholamine release (epinephrine and norepinephrine) [157]. Duration and intensity of exercise are the main factors able to stimulate SNS and alter catecholamine responses to exercise [157]. It is therefore biologically plausible that exercise-induced adrenergic-receptor stimulation has both acute (activation of UCP-1, stimulation of lipolysis) and chronic (UCP-1 gene transcription, mitochondrial biogenesis, hyperplasia of BAT, recruitment of brown adipocytes in WAT) effects on BAT.

Cardiac natriuretic peptides

Natriuretic peptides are hormones produced by the heart. Traditionally known actions of natriuretic peptides are natriuresis, diuresis, and vasodilation, which together serve to counteract excessive cardiac wall stress. However, receptors for the natriuretic peptides are not restricted to the kidneys and vasculature, but fat tissue is also rich in the receptors that bind atrial natriuretic peptide and B-type natriuretic peptide, as well as the receptors that promote their clearance [158]. Natriuretic peptides increase cyclic GMP levels to activate cGMP-dependent protein kinase, which shares homology with protein kinase A, activating p38MAPK [158]. These observed effects attributed to natriuretic peptides might be additive to, and perhaps synergistic with, those increases seen with classical β-adrenergic stimulation [159]. Bordicchia et al. [158] showed that in human adipocytes, natriuretic peptides induced lipolysis and UCP-1 expression, mitochondriogenesis, and increased uncoupled and total respiration [158]. Brain type natriuretic peptide treatment in mice enhanced EE and increased thermogenic protein levels in WAT and BAT [158].

Acute exercise increases the secretion of atrial and ventricular natriuretic peptides [160]. The stimulus for their secretion is the increase in heart rate as well as the stretch on atrial cardiomyocytes; therefore, atrial natriuretic peptides increase rapidly after the initiation of exercise [160,161]. The effects of long-term exercise effects on atrial natriuretic peptides in adults, and its role on human BAT activity and recruitment remain to be investigated. Moreover, whether the potential effects attributed to natriuretic peptides might be additive to, and perhaps synergistic with those increases seen with classical β-adrenergic stimulation needs to be studied.

FNDC5 expression and irisin release

Boström et al. [162] showed that murine skeletal muscles, upon increased levels of PGC-1α, induce the expression of a protein called FNDC5, which after cleavage is secreted into the blood stream as irisin. Irisin binds to the surface of white adipocytes, induce the expression of UCP-1, and triggers the transformation of white fat cells into beige cells [162].

Boström et al. [162] also showed an increased FNDC5 expression after 10 weeks of endurance exercise in obese male type 2 diabetic patients aged 53 years. More recently, Lee et al. [124] showed in humans that circulating irisin increases by 3 fold after 60 min of cycling at moderate intensity (40% VO2max). Interestingly, they observed no increases in irisin plasma concentration after a maximal exercise test, suggesting that there might be

a dose-response effect. In contrast, Timmons et al. [163] found no evidence that FNDC5 expression was increased after exercise in humans, yet, they showed that a group of old active participants had a 30% greater FNDC5 expression than sedentary controls. In summary, it seems that circulating irisin levels are up-regulated after exercise in humans yet there are still some inconsistencies which warrant further investigation [164,165]. Indeed, there are doubts on whether commercial methods are able to detect irisin as well as if irisin is really functional on humans [164]. Despite irisin seems to have been definitely detected in humans [165,166], the low serum concentration of the detected irisin peptide could make it physiologically ineffective, and thus questioning its possible effect when it is released by exercise.

Nevertheless, the FNDC5 gene is not only expressed in muscle, but also in WAT [167]. Moreover, its regulation in WAT could be sometimes antagonistic to the regulation in skeletal muscle [167]. Therefore, FNDC5 could regulate the browning process, not through the secretion of irisin, by through paracrine and autocrine action in WAT.

Interleukin-6

IL-6 is a multifunctional cytokine produced by immune (e.g. T cells) and non-immune cells, mainly adipose tissue and skeletal muscle [168]. IL-6 acts on a wide range of tissues through modulation of cell growth and differentiation [168]. For instance, in WAT, IL-6 increases lipolysis [169], whereas in skeletal muscle it increases glycolysis and improves insulin sensitivity [170]. On the other hand, it has been documented a relation between IL-6 and BAT metabolism. In rats, overexpression of the IL-6 gene increases thermogenic gene expression and elevates protein levels of UCP-1 in BAT, which is mediated by phosphorylation of STAT3 [168,171].

Stanford et al. [86] showed that the beneficial effects of murine BAT transplantation into WAT depots (e.g. improvement of glucose homeostasis and insulin sensitivity, weight loss) were blunted when BAT came from IL-6 knockout mice. This suggest that IL-6 is indeed required to maintain the profound metabolic effects of BAT transplantations and suggests that BAT-derived IL-6 could be a key factor acting as an autocrine or paracrine agent.

Exercise can promote IL-6 serum increases as significant as 100 fold [172]. Indeed, it seems that exercise intensity and duration, the form of muscular contraction (eccentric or concentric) and muscle damage are the main mechanisms that mediate the IL-6 response to acute exercise [173]. Taking into account that IL-6 is importantly regulated by exercising muscle and that BAT metabolism seems to be crucially mediated by IL-6, there is a need for human studies investigating the association between IL-6 increase in response to exercise and BAT activation and recruitment, and UCP-1 induction.

β-aminoisobutyric acid

Early in 2014, Roberts et al. [133] reported that BAIBA levels in muscle cells are regulated by PGC-1α and increases the expression of brown adipocyte-specific genes. In addition, exposure of human induced pluripotent stem cells to BAIBA during differentiation to mature white adipocytes induced a brown adipocyte-like phenotype. BAIBA also induced increased expression of brown/beige adipocyte-specific genes in vivo and muscle specific PGC-1α expression.

They also showed that 20 weeks of highly controlled endurance exercise training increased plasma BAIBA levels by 17% [133]. BAIBA also decreased weight gain and improved glucose tolerance in mice. Furthermore, BAIBA increased the expression of the browning gene program through a specific PPARα-dependent mechanism on white adipocytes in vitro and in inguinal white fat depot of mice. Finally, BAIBA plasma concentrations were inversely correlated with cardiometabolic risk factors in a large human cohort study (Community-based Framingham Heart Study) and were increased during exercise training in subjects of the HERITAGE Family Study.

Fibroblast growth factor 21

FGF21 is one of the "hormone-like" members of the fibroblast growth family. It is mainly expressed by the liver, but also by other tissues as the thymus, WAT and skeletal muscle [174,175], as well as by BAT [176]. FGF21 can act on BAT as an autocrine, paracrine and endocrine agent, activating BAT thermogenesis and UCP-1 expression. On WAT, FGF21 induces the "browning" process by enhancing adipose tissue PGC-1α protein levels [177,178]. Hanssen et al. [179] showed that circulating FGF21 levels was associated with BAT activity during acute cold exposure and that cold acclimation increased BAT activity in parallel with increased FGF21 levels in men.

Catoire et al. [180] found that FGF21 muscle expression was induced after 1 h one-leg cycling, while no variation was found in the non-exercising leg. Interestingly, Kim et al. [181] found no variation in serum FGF21 levels immediately after the exercise protocol but after one hour of recovery. On the other hand, Cuevas-Ramos et al. [126] found no acute effect of exercise on serum FGF21 but an increase concentration after a 2 weeks of a combined training program. These findings contrast with those found by Scalzo et al. [182], who reported a decrease of FGF21 expression in muscle after three weeks of sprint interval training. Lee et al. [124] also failed to find any effect of exercise on FGF21, and they even observed a non-significant decrease in serum levels of FGF21. Several animal studies suggested that FGF21 induction by exercise could be dependent of the studied tissue. Kim et al. [181] found an increase in the hepatic FGF21 expression while they did not find induction in skeletal muscle. It has even been postulated that exercise benefits related to FGF21 are explained by an increased hepatic sensibility to FGF21.

Meteorin-like

The expression of a splice form of the gene encoding PGC-1a, termed PGC-1a 4 promotes muscle hypertrophy and strength, and regulates EE in mice and humans in response to resistance training [183]. Importantly the overexpression of PGC-1a 4 stimulates the expression and secretion of a protein called meteorin-like [131]. Upon binding its receptor in adipose tissue, meteorin-like promotes an eosinophil dependent activation of M2 macrophages, secreting IL-4 and IL-13, which in turn induces WAT browning and induce the expression of genes encoding thermogenic and mitochondrial program [131,135]. Meteorin-like is also secreted in adipose tissue [184]. Importantly, administration of an anti-meteorin-like antibody partially prevented cold-induced WAT browning [185]. Only few preliminary studies have been conducted regarding the role of meteorin-like in the browning process, and therefore its regulating role deserves further investigation [135].

Rao et al.[131] showed that meteorin-like mRNA expression is induced in murine skeletal muscle after a resistance exercise session. This overexpression concurred with increased levels of circulating meteorin-like which remained elevated 24 hours after the exercise session. In contrast, Rao et al. [131] reported that a free wheel running (i.e. endurance exercise) did not modify circulating meteorin-like concentrations. Importantly, they also observed an increased in meteorin-like levels after a single session of combined exercise (i.e. endurance and resistance exercise) in humans [131]. Further studies are warranted to identify the factors (e.g. exercise intensity, age, body composition) modifying the meteorin-like secretion in humans.

Brain derived neurotrophic factor

The hypothalamic BDNF exerts its main function within the central nervous system, although it also acts on peripheral tissues, regulating energy homeostasis and immune function [186]. In mice, BDNF is secreted in response to an enriched environment (i.e. the presence of mazes and toys) and exercise, resulting in WAT browning in both cases [185]. Importantly, the artificial inhibition of BDNF during exercise inhibited the exercise induced WAT browning [187]. Crucially, BDNF effects seems to be, at least in part, exerted by means of the expression of PGC-1α and FNDC5 [188]. Nevertheless, circulating BDNF levels are upregulated in response to exercise [128,189], and therefore, constitute a plausible mechanism of the exercise-induced WAT browning.

Myostatin

GDF-8, also called myostatin, is a member of the TGF-β superfamily. This was described to be a myokine early in the 1990s [190]. Myostatin´s main function is the inhibition of muscle growth, and consequently, its suppression dramatically stimulate muscle growth [191]. Myostatin loos of function not only results in muscle hypertrophy, but also in decreased fat accumulation [192], and fat browning [193,194]. The induction of fat browning by myostatin inhibition is triggered by the activation of the AMPK enzyme and the subsequent induction of PGC-1a and FNDC5 [195]. Therefore, myostatin seems to play an important role as WAT browning inhibitor. Moreover, it has been recently shown that BAT-muscle connection through myostatin could be bidirectional, with BAT influencing muscle function by secreting myostatin [70].

Acute and chronic exercise modify myostatin expression and circulating levels, although this effect seems to be dependent on the type and intensity of exercise [196–199]. Kabak et al. [196] showed that myostatin levels during resting were similar in a group of professional kick-boxers than in a group of sedentary counterparts. Moreover, high-intensity exercise decreased the myostatin serum concentration of both groups during at least 3 hours [196]. Interestingly, another study showed that this-exercise increased myostatin level is related to the dietary protein intake [199]. In contrast, Saremi et al. [198] found that an 8 weeks resistance training program decreased resting myostatin levels, which concur with the decrease in serum myostatin concentration 24 hours after a resistance training session observed by Kazemi et al. [197].

Importantly, the myostatin effect on BAT represent a proof of concept of that exercise, at least some types of exercise, induce the secretion of not only pro-browning agents, but also browning inhibitors. Among these mechanisms possibly mediating the

plausible BAT inhibition in response to exercise, others molecules, such as LR11/SorLA (sLR11) [200], should be further studied.

Follistatin

Follistatin binds several members of the TGF-β superfamily including activins and myostatin to neutralize their biological activities [201]. Follistatin can be secreted by muscle, liver, and other tissues including WAT and BAT [201]. Therefore, the suppression of the myostatin-signaling has been identified as an important pathway involved in muscle metabolism, differentiation, and growth [202]. Moreover, follistatin expression, either in an autocrine or endocrine manner results on WAT browning stimulation. Interestingly, follistatin levels have correlated to irisin levels [135].

Exercise modifies follistatin circulating levels. For instance, Perakakis et al. [202] found that two different exercise intensities (i.e. 70% and 90% of VO_2max) acutely increased follistatin levels, independently of the presence of the metabolic syndrome in the studied participants. Another study showed that follistatin levels are hardly modified after an eccentric exercise in young adults [203]. In contrast, Sargeant et al. found that circulating follistatin levels were elevated after a moderate-intensity bout of exercise (i.e. 60 minutes at 60% VO_2max) and remained elevated for at least 6 hours [204].

Musclin

Musclin is a peptide produced by skeletal muscle as a response to exercise and it can be found in bloodstream [121]. Musclin mRNA expression has been linked to insulin-induced activation of Akt that phosphorylates FOXO1, causing it to be exported from the nucleus and thus releasing the musclin-encoding gene from transcriptional inhibition [132]. Musclin shares some structural similarities with natriuretic peptides, and consequently, can bind to some common receptors [132]. Musclin promotes mitochondrial biogenesis and exercise endurance in skeletal muscle [132]. Although it is clear that musclin is secreted in response to exercise in murine models, whether it is also the case in humans remains to be elucidated. Nevertheless, since musclin works as a PPARγ agonist it has been suggested to play a role on the browning process [121]. Future studies are needed to confirm this hypothesis.

Leptin

Leptin was discovered in 1994 [205]. Leptin is mainly produced and secreted by WAT, and indeed leptin serum concentrations are tightly correlated with fat mass [206]. Leptin regulates energy homeostasis, both by means of suppressing appetite and stimulating EE, binding the hypothalamus [207]. The stimulation of EE take place in part by increasing sympathetic activation of BAT [208]. Moreover, leptin deficiency results in impaired BAT function [209]. Besides the hypothalamus, leptin is also targeting skeletal muscle, where it stimulates muscle growth and oxidative metabolism, and importantly stimulate FNDC5 expression [167]. It potentially would lead to increased irisin secretion; however, it does not seem to be the case [167]. Paradoxically, in opposition to its effect on skeletal muscle, leptin downregulates the expression of FNDC5 in WAT [167], which is translated to inhibition of UCP-1 expression and beige adipocytes recruitment [167].

The effects of exercise over leptin concentrations are controversial. Some studies have reported decreased leptin level after an exercise training program, although this effect may be dependent on fat mass changes [210]. Acutely, physical exercise have been reported to induce a decrease in leptin circulating concentrations [211], although not consistently [126,212].

Adiponectin

Adiponectin is a cytokine largely secreted by WAT, but not by BAT [69]. Adiponectin secretion is inversely related to BMI, stimulate glucose utilization, fat oxidation in skeletal muscle and is inversely related to insulin resistance [213]. Importantly, adiponectin secretion in WAT can be stimulated by cold exposure and contribute to the recruitment of M2 macrophages [214], which would contribute to the long-term browning of WAT depots [69].

The effect of exercise on adiponectin circulating levels has provided controversial results. Some studies did not find any change after an exercise training program [130,215], although others suggested that the chronic effect of exercise over the adiponectin serum concentration may be intensity-dependent [213]. Acute exercise seems to increase adiponectin concentrations during the recovery period [211,212], although it seems to rapidly return to baseline levels [126,216].

Metabolites: Lactate and β-hydroxybutyrate

Lactate is a metabolite largely produced by the high intensity exercising-muscle as a product of anaerobic glycolysis. Brown adipocytes overexpress the monocarboxylic transporter I, a lactate transporter into cells, in response to exercise [217]. As a consequence, lactate-induced browning of WAT is thought to be mediated by a change in intracellular redox state (NADH-to-NAD+ ratio) [154]. However, the lactate induced WAT browning may not be completely explained by this direct mechanism, since it seems to induced FGF21 expression in brown adipocytes, which likely acts in an autocrine manner to induce browning [218].

Similarly to lactate, the ketone body β-hydroxybutyrate seems to induce WAT browning, also by a change in intracellular redox state (NADH-to-NAD+ ratio) [154]. Ketone bodies such as β-hydroxybutyrate are used as fuel source when glucose availability is reduced [219]. During exercise, β-hydroxybutyrate circulating concentrations are commonly decreased, as a consequence of a higher muscle uptake than hepatic production [219]. Nonetheless, it is common to observe increased circulating levels of β-hydroxybutyrate during prolonged exercise or after intense exercise [219–221]. Therefore, it might be that the hepatic release of β-hydroxybutyrate to comply with energy requirements during and after exercise could also signal in WAT to induce browning.

In summary, there is a growing number of endocrine factors that could link exercise metabolism to BAT/beige metabolism (Figure 1), providing potential mechanistic explanation to both the increased in WAT browning and the inhibition of classical BAT in response to exercise.

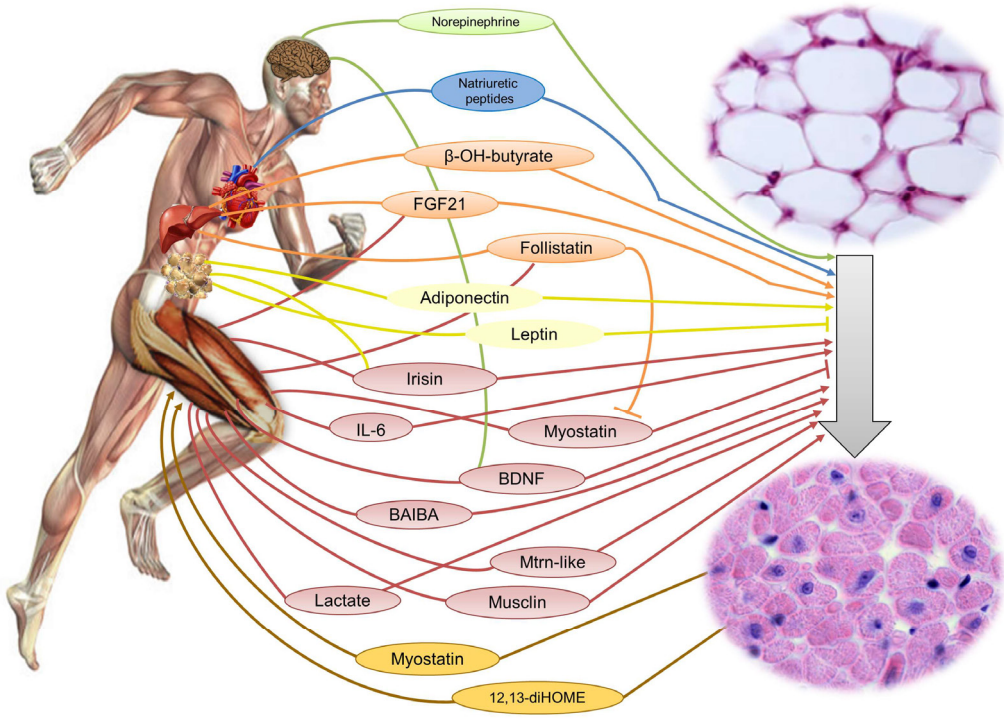

Figure 1. Endocrine regulation of white adipose tissue (WAT) browning during exercise. During exercise the central nervous system orchestrate the secretion of norepinephrine, which is the main activator of brown adipose tissue (BAT) and WAT browning. In addition, the central nervous system contributes, together with skeletal muscle, to increase the levels of the hypothalamic brain derived neurotrophic factor (BDNF) which can signal in WAT activating the browning process by activating the fibronectin type III domain containing 5 (FNDC5) expression. During exercise, the circulating levels of natriuretic peptides (atrial and ventricular) are elevated as a consequence of the increased secretion from the cardiac muscle. The natriuretic peptides can activate BAT thermogenesis and WAT browning to similar mechanism than norepinephrine. The liver is also a relevant secreting organ during exercise. Together with skeletal muscle secretion, the liver is responsible of elevating circulating levels of fibroblast growth factor 21 (FGF21) and Follistatin during exercise. FGF21 can directly bind with adipocytes to induce the browning program. Follistatin acts inhibiting myostatin function, which consist on inhibiting both muscle growth and the browning process. The ketone boy β-hydroxybutyrate (β-OH-butyrate) is used as an energy source by muscle during exercise. However, its circulating level is increased during prolonged exercise and after intense exercise due to increased hepatic production. During exercise, the skeletal muscle secretes several peptides [i.e. Irisin, Interleukin-6 (IL-6), β- Aminoisobutyric acid (BAIBA), Meteorin-like (Mtrn-like), and Musclin], that together with the previously mentioned BDNF and FGF21 can induce the browning process in WAT. Skeletal muscle also secretes important quantities of lactate during intense exercise, which also can signal in WAT activating browning. Finally, BAT also secretes molecules [myostatin and 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME)] able to regulate muscle function.

In humans, only few preliminary studies have been conducted examining the effect of exercise on BAT or WAT browning. Among them, six observational studies have shown controversial results [136–138,222,223]. Dinas et al. [136] showed for the first time a promising association between self-reported habitual PA and BAT activity in a sample of forty (14 females) patients with cancer. Results are however limited due to the fact that they assessed habitual PA with a questionnaire, which has low accuracy [224]. In contrast, we failed to observed any association between PA levels, objectively measured by accelerometry during 7 days, and BAT volume or ^{18}F-FDG activity, after a personalized

cold exposure, in young healthy adults [225]. On the other hand, three independent research groups have shown that BAT ^{18}F-FDG uptake is lower in endurance-trained individuals of both genders than in sedentary counter-parts [137,138,223]. Vosselman et al. [138] conducted a case-controlled study where they compared BAT activity as well as browning of subcutaneous abdominal WAT in male endurance-trained (i.e. runners, cyclist and swimmers with a VO2max >55 ml/kg/min, and with a training experience of at least 2 years) with an age- and BMI-matched group of sedentary lean males. They observed that BAT activity was significantly lower in the endurance-trained group compared with their sedentary counterparts. Later, Singhal et al. [137] showed similar results than Vosselman, but in a group of endurance trained women. Another study also incidentally found less BAT volume and activity, assessed by ^{18}F-FDG, in 2 trained participants than in a group of 10 sedentary counterparts [223].

Moreover, contradictory findings are also shown regarding the expression of beige marker in WAT. Dinas et al.[222] showed that participants reporting moderate PA levels had higher expression of browning markers (PGC-1α, PPARα or PPARγ) in abdominal subcutaneous WAT in comparison to participants who reported low levels of PA, whereas levels of UCP1 were similar. In contrast, Vosselman et al.[138] did not find any statistical difference in abdominal subcutaneous WAT browning markers (i.e. UCP-1, PGC-1α, Cidea, TMEM26 or CD137) expression between trained and untrained men. Interestingly, they observed that mRNA expression of FNDC5 in skeletal muscle (vastus lateralis) was 1.6-fold higher in the endurance-trained group, suggesting that long-term endurance exercise stimulates FNDC5 expression.

In addition to observational studies, three experimental studies have been published, one studying insulin-stimulated BAT ^{18}F-FDG uptake [226], and two studying expression of browning markers in abdominal scWAT [227,228]. Insulin-stimulated BAT ^{18}F-FDG uptake was reduced after 6 sessions (2 weeks) of cycling training, only when combining two groups of different training modalities (i.e. High intensity interval and moderate intensity continuous training) [226], in those participants with high BAT glucose uptake in the baseline (n=6), but not in low-BAT individuals (n=12). In contrast, the free fatty acids uptake was decreased in low-BAT individuals and not modified in high-BAT individuals. However, the lack of a control group, the short duration of the training program, and the small sample size, precludes from drawing definitive conclusions [226]. Regarding expression of beige markers, one study did not find any effect after 6 weeks of endurance training [228], while other found an induction effect when combining data of pre-diabetic and normo-glycemic groups (importantly, this study did not include a control group and therefore browning markers expression could be mediated by seasonal effect) after 12 weeks of combined endurance and strength training [227].

As mentioned earlier, exercise seems to differentially regulate classical BAT and WAT browning in mice. However, it should be considered that due to the much bigger surface/volume ratio, BAT metabolism in rodents is much more relevant than it is in humans [3]. Moreover, histological and molecular studies have revealed that the cold inducible adipose tissue in humans is not identical to neither murine classical BAT, nor beige [229], which adds extra difficulties for transferring the murine physiology into humans. In summary, there is epidemiological evidence [136–138,222,223], preliminary experiments [226,227] and possible mechanism [28,135,185] suggesting both an stimulatory and an inhibitory effect of

exercise on human BAT activation and/or WAT browning. If the BAT inhibition hypothesis is real, this BAT adaptation to exercise could partially explain, by means of a reduced adaptive thermogenesis, the observed decrease in non-muscular EE hypothesized by Pontzer et al. [7] in the Constrained TEE model. Indeed, in a different study, Pontzer et al. [230] reported that the PA-TEE relationship in a group of Bolivians farmers was less concordant with the Constrained model than in Tanzanians hunter-gatherer and U.S. people. Farmers from the alteplano of Bolivia are likely to be exposed to low temperatures, and thus, it could be that cold-induced BAT activation (i) counteracts the reduction in non-muscular EE and (ii) enhances TEE in highly active individuals. More studies are needed to test these hypotheses, and if confirmed, new strategies have to be designed to counteract the BAT "side effect" of exercise-based therapies for preventing and treating obesity and related comorbidities.

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AIMS

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The overall aim of this Doctoral Thesis is to study the role of human brown adipose tissue in energy balance and metabolism regulation, and to study the effect of exercise on BAT volume and activity, as well as in energy balance related variables in humans. This overall aim is addressed in eight studies. In addition, the Doctoral Thesis also contains two methodological studies that were conducted to solve some methodological aspects to be applied in the rest of studies.

Methodological section

- General methodological objective: To determine the best methods for data selection and analyses for assessing basal metabolic rate and cold-induced thermogenesis in healthy humans.
    - Specific methodological objective 1: To analyze the impact of methods for data selection on basal metabolic rate and respiratory exchange ratio estimations, and in their inter-day reliability in young health adults (**Study 1**).
    - Specific methodological objective 2: To analyze the impact of methods for data selection and methods for data analysis on cold-induced energy expenditure and nutrient oxidation rates estimations in young health adults (**Study 2**).

Section 1

- General objective 1: To investigate the relationship between human brown adipose tissue volume and activity and energy balance and metabolism regulation.
    - Specific objective 1.1: To describe the energy expenditure and nutrient oxidation response to an individualized non-shivering cold exposure in young adults (**Study 3**).
    - Specific objective 1.2: To examine the associations of brown adipose tissue and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold exposure with cold-induced energy expenditure and nutrient oxidation rates in young adults (**Study 4**).
    - Specific objective 1.3: To examine the associations of brown adipose tissue and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold exposure with basal and post-prandial energy expenditure and nutrient oxidation rates in young adults (**Study 5**).
    - Specific objective 1.4: To examine the association of brown adipose tissue and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold exposure with energy intake and appetite-related sensations in young adults (**Study 6**).

Section 2

- General objective 2: To investigate the relationship between human BAT volume and activity and body composition.
    - Specific objective 2.1: To examine the association of brown adipose tissue and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold exposure with lumbar and whole-body mineral density in young adults (**Study 7**).
    - Specific objective 2.2: To examine the association of brown adipose tissue and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold exposure with body mass index and body composition (lean mass, fat mass and visceral adipose tissue mass) in young adults (**Study 8**)

Section 3

- General objective 3: To investigate the role of BAT volume and activity, and energy balance regulation in the development of the metabolic syndrome.
    - Specific objective 3.1: To compare brown adipose tissue and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold exposure, and energy balance related variables between metabolically healthy overweight or obese and metabolically unhealthy overweight or obese young adults (**Study 9**).

Section 4

- General objective 4: To investigate the role of physical exercise on brown adipose tissue and energy balance regulation.
    - Specific objective 4.1: To study the effect of a 6 months combined exercise training program on brown adipose tissue and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold exposure, energy balance related variables and body composition in young adults (**Study 10**).

METHODS

The activating brown adipose tissue through exercise (ACTIBATE) study: Design and methodology

STUDY

DESIGN

The ACTIBATE study is a randomized controlled trial (ClinicalTrials.gov ID: NCT02365129). The Human Research Ethics Committee of both University of Granada (n° 924) and Servicio Andaluz de Salud (Centro de Granada, CEI-Granada) approved the study design, study protocols and informed consent procedure. All participants had to provide a written informed consent. Participants were randomly allocated to the usual care (control), moderate-intensity exercise or vigorous-intensity exercise groups and were followed for 6 months during the exercise interventions. All the baseline and follow-up examinations were performed in the same setting [Instituto Mixto Deporte y Salud (iMUDS) at the University of Granada and Hospital Universitario Virgen de las Nieves, Granada, Spain] and by the same investigators. The study was performed following the ethical guidelines of the Declaration of Helsinki, last modified in 2013. The baseline evaluations were performed between October and November 2015 (n≈60 participants) and 2016 (n≈90 participants).

Participants and selection criteria

Eligible participants should be 18-25 years old and should have a BMI between 18.5 to 35 kg/m^2. The inclusion and exclusion criteria are listed in Table 1. Including people with different weight status and body composition allows to study BAT differences across categories (i.e. normal-weight, overweight and obese based on BMI, and on body fat percentage). BAT seems to be related to age and BMI [1,2], being higher in younger and thinner people. Thus, people aged 18-25 years old are expected to present detectable BAT volume and activity [3,4]. Further, persons of this age are legally able to sign themselves informed consent to participate in ACTIBATE, and no ethical issues are raised due to participant's age and type of measurements to be undertaken in the study.

Table 1. Selection criteria.

Inclusion criteria	Exclusion criteria
-Age: 18-25 years. -BMI: 18.5-35 kg/m^2. -Not engaged in regular physical activity >20 min on >3 days/week. -Not participating in a weight loss program. -Stable weight over the last 3 months (body weight changes <3 kg). -Normal electrocardiogram. -Participants must be capable and willing to provide consent, understand exclusion criteria and accept the randomized group assignment.	- History of cardiovascular disease. - Diabetes or hypertension. - Pregnancy or planning to get pregnant during the study period. -Medication for hypertension, hyperlipidemia, hyperuricemia or other illness. -Beta blockers or benzodiapezins use. -Smoking. -Frequent exposure to cold temperatures (Granada is surrounded by high mountains where people can ski or do trekking). -Taking medication for thyroid. -Other significant medical conditions that are life-threatening or that can interfere with or be aggravated by exercise. - Unwillingness to either complete the study requirements or to be randomized into control or training group. - A first-degree relative with history of cancer.

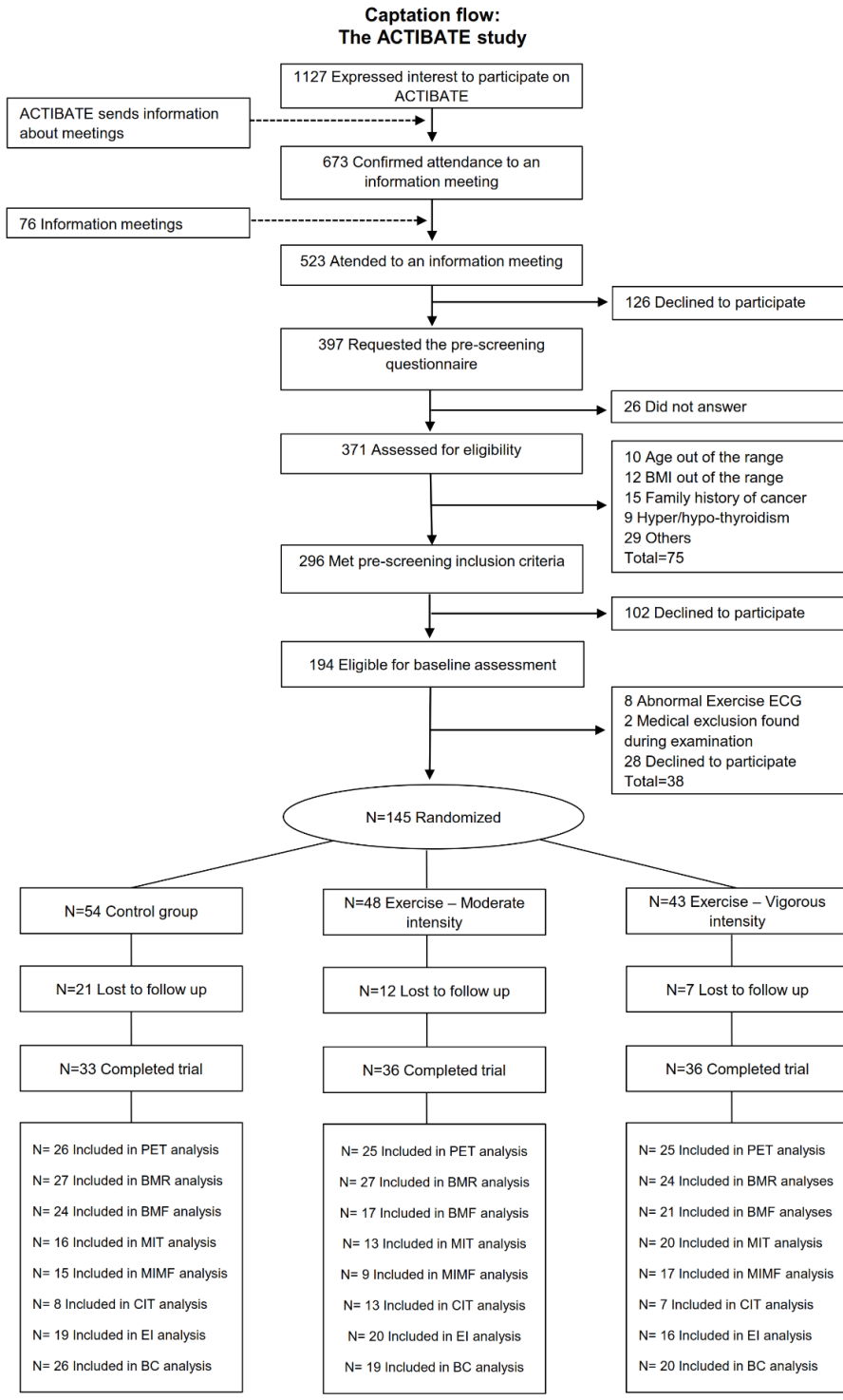

Figure 2. Captation flow of the activating brown adipose tissue through exercise (ACTIBATE) study. ECG: Electrocardiography; PET: Positron emission tomography-computerized tomography; BMR: Basal metabolic rate; BMF: Basal metabolic flexibility; MIT: Meal-induced thermogenesis; MIMF: Meal-induced metabolic flexibility; CIT: Cold-induced thermogenesis; EI: Energy intake; BC: Body composition.

Risks were minimized by ruling out contraindications to the testing and training protocols via a health history and a thorough physical examination prior to the testing sessions. If any medical problems appeared during the project, participants were referred for medical evaluation and, if necessary, dropped from the study. ACTIBATE subscribed private liability insurance covering participants, investigator's responsibility as well as the responsibility of any person involved in the study, provided that there is proper adherence to the protocol.

The study was announced on social networks, local media and posters at the Faculties of the University of Granada. We also organized information meetings at the different faculties (e.g. Nurse, Nutrition, Computer Science, Engineering). People interested contacted the research team through e-mail, Facebook or Twitter. Later, they visited the research center to receive a thorough explanation about the study aims, measurements to be undertaken, study requirements of the participants, and intervention types. What participants can expect from the study was clarified, and any questions were answered. Thereafter, potential interested participants received a short web-based questionnaire to obtain information about age, weight, height, PA, weight during the previous 3 months, current medical history and medication use, smoking and alcohol habits, and residence. Individuals who remained eligible after the first screening and expressed willingness to participate in the study were invited to a second orientation session. In the second visit, participants received detailed written information about the study. Eligible participants who wished to participate in further screening were asked to sign the informed consent. Participants were then scheduled for their first baseline measurement visits. Figure 2 illustrates the participant flow from recruitment to follow-up.

Randomization and blinding

Eligible participants were randomly assigned after completing the baseline measurements to either the control or exercise training groups. The randomization was computer generated. We used simple (unrestricted) randomization [5]. Assessment staff were blinded to participant randomisation assignment. Participants were explicitly informed on the group to which they had been assigned. For practical and feasibility reasons, the study was conducted in waves of 16 or 24 persons.

Sample size

The estimations of the sample size and power of our study were made based on diverse observations from cold exposure studies and β3-adrenergic receptor agonists in humans, and taking assumptions extrapolated from animal models [6–8]. We used a conservative approach to sample size estimation and assumed a relatively large SD based on the heterogeneity of data published in humans at the time the study was planned (2011-2012). Moreover, due to the nature and duration of the study as well as the complexity of the outcomes to be measured, there were conservative estimates of effect sizes, accounting for higher drop-outs than that achieved in prior exercise-based randomized controlled trials. Power calculations were made for the primary outcome measures of BAT volume.

We anticipated modest increases of 10% and 20% on activated BAT volume at 6 months in moderate-intensity and vigorous-intensity exercise groups, respectively, from a baseline level of 50-70 g [2,9,10], and change score SD of around 50-60 g. The anticipated

increases in BAT activity were much lower than those observed in cold exposure or β3-adrenergic receptor agonists studies [6–8]. Assuming a bilateral alternative (which means that an effect in either direction will be interpreted), we can detect differences of at least 10% in BAT volume with a power of >80% and α of 0.05 in a group of 17 participants. Since several studies reported sex-differences in BAT volume [2], it was planned to perform analyses in men and women separately. To avoid loss of statistical power, a total of 34 (50% women) participants should be enrolled in each group. Assuming a maximum loss at follow-up of 30%, we decided to recruit 50 participants (≈50% women) for each of the study groups: control, moderate-intensity and vigorous-intensity groups. A total of 150 participants were planned to be enrolled in ACTIBATE. We used IBM-SPSS Sample power software (version 3.0.1) for calculations.

Participant retention and adherence

Participants were allowed to withdraw at any time. However, to reduce participants drop out and to maintain adherence to the training program, several strategies were used (see below). In anticipation of private commitments, vacations, etc. that might interfere with a participant's availability to come to the exercise centre for an exercise session, participants were allowed to do exercise out of the centre. In this situation, participants received a heart rate monitor and instructions on how to operate it, a kit of elastic bands to perform the strength training and the session planning. They were asked to complete a detailed log sheet to record the type of activity completed and duration of exercise.

All sessions were accompanied with music that participants chose, and were performed on an airy, well-lighted exercise room. Qualified fitness specialists carefully supervised every training session and worked with groups of no more than 15 persons to ensure that participants were performing the exercises correctly and at proper intensity. The training specialist and other study staff constantly supported participants.

EXERCISE PROGRAM AND SELECTION OF DOSES

With the final aim of making the exercise program transferable to society, the basis for the specific exercise dose in ACTIBATE was the PA recommendations for adults proposed by the World Health Organization [11]. Since there is no information regarding the ideal exercise model to activate and recruit BAT, ACTIBATE combined both aerobic and resistance training. A major objective of the study is to evaluate various exercise intensity levels (moderate and vigorous) that fall within the current public health recommendations to test whether higher intensity levels provide more benefit than the standard moderate-intensity level.

The length of the trial is 6 months based on results from previous large scale randomized controlled trials and based on the fact that substantial physiological adaptations occur within the first 3-6 months of exercise [12,13]. We also considered the increased logistical and participation burdens, which indeed might lead to poorer adherence, as well as the cost of running a highly controlled-laboratory based study for a longer period. No dietary prescription or instructions were provided to the participants in both control and exercise groups, except for the prescription to not change their dietary habits during the study period.

Volume

An important goal in the development of exercise doses of ACTIBATE was to keep the total volume (in minutes) of weekly exercise similar in both intervention groups while ensuring that the aerobic, as well as the resistance training, prescriptions met current guidelines [11,14]. We achieved both goals since the total time of aerobic exercise in both moderate-intensity and vigorous-intensity groups was 150 minutes/week, whereas the time needed to complete the resistance training exercises was ≈80 minutes for both groups.

It is estimated that 150 minutes/week of moderate-intensity [≈3-5.9 METs; 1 MET= 3.5 ml O_2/kg/min] aerobic PA is equivalent to 1000 kcal/week, which is associated with lower rates of cardiovascular disease and premature mortality [15]. An EE of 1000 kcal/week can also be achieved with ≈75 minutes/week of vigorous intensity (≥6 METs).

We had discussions on whether the total volume of expended energy in both moderate-intensity and vigorous-intensity groups should be equal, so that it would be possible to test the independent contributions of volume expended versus intensity. The volume selected for ACTIBATE is based on the minimum weekly PA time recommended by the public health organizations, with the final goal of making the results of the intervention easily transferable and understandable to the population in terms of time in minutes/week, intensity, and frequency. The lack of time is one of the main reasons reported to justify not to engage in PA. For this reason, we decided to test two different training strategies that take the same time, but that due to the different intensities might lead to different results. Thus, both the moderate-intensity and vigorous-intensity groups performed 150 minutes/week of aerobic exercise and ≈80 minutes/week of strength training. For the aerobic exercise, the vigorous intensity group performed 75 minutes/week at moderate intensity (i.e. 60% HRres) and 75 minutes/week at vigorous intensity (i.e. 80% HRres), while the moderate-intensity group performed the total of 150 minutes/week of aerobic training at 60% HRres. The strength training was performed at 50% of 1 RM for the moderate-intensity group and at 70% RM for the vigorous-intensity group. We monitored exerciser's heart rate (RS800CX, Polar Electro Öy, Kempele, Finland) during the exercise sessions. Additionally, rating of perceived effort was collected each session [16].

Intensity

Several public health institutions recommend that moderate-intensity PA might be beneficial for health in deconditioned persons [11,14,15]. Yet, additional benefits have been observed of vigorous vs. moderate-intensity exercise [15]. An intensity of 60% HRres is sufficient to produce clinically significant physiological adaptations in sedentary individuals [11,14,15]. The intensity selected for ACTIBATE was 60% HRres for the moderate-intensity group and 80% of HRres for the vigorous-intensity group. The intensity for the resistance training was 50% RM and 70% RM for the moderate-intensity and vigorous-intensity group, respectively. 1RM is the maximum amount of weight one can lift in a single repetition for a given exercise. An intensity equivalent to 40-50% of 1RM may be beneficial for improving muscle strength in sedentary persons beginning a resistance training program [17], whereas 60%-70% of 1RM is recommended for novice to intermediate exercisers to improve strength [17]. As the load (i.e. %RM) is not the only variable that influences strength training intensity, we controlled variables as speed of movement during both concentric

and eccentric phase, recovery time and range of motion. Therefore, we assume that different loads (i.e. 50% RM and 70% RM) really constituted different training intensities [17–19].

Frequency

Although the PA recommendations suggest doing PA on most, preferably all days of the week, there was a concern that more than 4 days per week would be an excessive burden and might have undesirable effects on adherence and motivation to the exercise intervention program. Studies on exercise frequency show little differences for 3 or more days per week provided the weekly dose of exercise is attained [15]. Participants in ACTIBATE were asked to do their weekly exercise dose in 3 to 4 sessions, at their own choice. The length of the training sessions was adjusted, so that participants attending 3 or 4 days/week had the same weekly dose. Strength training was performed on 2 of these 3 or 4 days/week, and therefore 1 or 2 sessions per week consisted solely on aerobic exercise. Participants were advised to not to be out of training for more than 2 consecutive days and were not allowed to train <3 days/week or >4 days/week. Participants were contacted if they missed a scheduled session or if they did not meet the weekly recommendations.

Type of exercise

Activities programmed for the aerobic exercise were cycle ergometer, treadmill and elliptical ergometer. The resistance training program mainly involved major upper and lower body muscle groups [17].

Training load variation

We were aware that participants might not be immediately capable of exercising at their required volume and intensity dose; therefore, there was a gradual progression to the assigned exercise dose (see Table 2).

For the aerobic training, participants of both exercise groups started with a dose of 75 minutes/week at 60% HRres. The volume was increased 30 minutes/week, so by the 4th week both groups achieved the 150 minutes/week dose. From this point onwards, the vigorous-intensity group started a gradual increase of the exercise intensity. For this group, half of the aerobic training time was performed at a higher intensity, which was 5% higher than previous week (i.e. 60% HRres the 4th week, 70% HRres the 5th week, etc.), so by the 7th week, both groups were training at the pre-specified dose.

For the strength training, participants went through a familiarization period during the first 4 weeks of the study. During this phase, participants learned the movement patterns that constitute the base of the different exercises (e.g. squat, horizontal pull, vertical push). They also performed compensatory training such as core stability, flexibility and stabilizers muscles in order to minimize risk of injuries as well as to promote training adherence.

Table 2. Training periodization; Grey blocks represent weeks where training load is reduced in order to learn the proper technique of exercised used in the next phase Black blocks represents weeks where strength training mostly consist on RM assessment in the different exercises used in

	Phases	Familiarization				Phase 1					Phase 2					Phase 3				Phase 4					
	Weeks	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Aerobic training	Aerobic training volume (min)	75	105	135	150	120	150	150	150	150	120	150	150	150	150	120	150	150	150	150	120	150	150	120	150
	Intensity (%HRres) moderate-int group	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60	60
	Intensity (%HRres) vigorous-int group	60	60	60	60	70	75	80	80	80	80	80	80	80	80	80	80	80	80	80	80	80	80	80	80
Strength training	Intensity (%RM) moderate-int group	Weight- bearing and elastics bands					50	50	50			50	50	50			50	50	50		50	50		50	50
	Intensity (%RM) vigorous-int group						50	60	70			60	70	70			60	70	70		70	70		70	70
	Number of different combined session per week (session type)	1					2 (A_1 and A_2)					2 (A and B)					2 (A and B)				2 (A and B)			2 (A and B)	
	Type of exercises performed	Slow and global movement pattern (light weights)					Exercises localized in mayor muscle groups					Exercises localized in mayor muscle groups					Whole body exercises				Exercises localized in mayor muscle groups			Whole body exercises	
	Training stimulus aim	Learning of movement patterns					Initial adaptations to resistance training					Session A: mechanical tension and muscle damage					Session A: mechanical tension and muscle damage				Session A: mechanical tension and power			Session A: mechanical tension and power	
		Compensatory training: -Core stability - Flexibility - Stabilizers muscles					Compensatory training: -Core stability - Flexibility - Stabilizers muscles					Session B: Metabolic Stress					Session B: Metabolic Stress				Session B: Metabolic Stress			Session B: Metabolic Stress	

We were aware that both aerobic and strength training load should be incremented as the participants fitness increased. Aerobic training intensity was controlled based on HRres, which means that a higher speed or power (i.e. external load) had to be selected to achieve a determined percentage of HRres when fitness was increasing. For the strength training, the load equivalent to the participant's 50% RM or 70% RM was assessed at the beginning of each training phase (i.e. each 5 weeks approx., see Table 2). On the other hand, it is well known that different configurations of the strength-training stimulus can elicit different physiological responses (e.g. muscle damage, metabolic stress, etc.) [20]. Taking into account that it is not known which kind of strength training stimulus is better to activate or recruit BAT, the training program varied the type of stimuli across the different training phases.

Table 3. Different types of combined training session. Regarding to strength training, session type A is aiming to maximize mechanical tensions and muscle damage, while session type B is aiming to maximize metabolic stress. On session type A, strength exercise order alternate between upper-body and lower-body, and agonist and antagonist exercises, allowing the maximum recovery possible to the implied muscle groups. On session type B, all upper-body exercises are performed before the lower-body exercises, and all agonist muscle groups exercises are also performed afterward each other.

SESSION TYPE A		
Warm-up		
MAIN PART	**Exercise**	**Time/sets**
	Aerobic set 1	10 min
	Strength exercise A	1set x 10reps
	Strength exercise B	1set x 10reps
	Strength exercise C	1set x 10reps
	Strength exercise D	1set x 10reps
	Aerobic set 2	10 min
	Strength exercise E	1set x 10reps
	Strength exercise F	1set x 10reps
	Strength exercise G	1set x 10reps
	Strength exercise H	1set x 10reps
	Aerobic set 3	10 min
	Strength exercise A	1set x 10reps
	Strength exercise B	1set x 10reps
	Strength exercise C	1set x 10reps
	Strength exercise D	1set x 10reps
	Aerobic set 4	10 min
	Strength exercise E	1set x 10reps
	Strength exercise F	1set x 10reps
	Strength exercise G	1set x 10reps
	Strength exercise H	1set x 10reps
Cooling down		

SESSION TYPE B		
Warm-up		
MAIN PART	**Exercise**	**Time/sets**
	Strength exercise A	2sets x 10reps
	Strength exercise G	2sets x 10reps
	Strength exercise E	2sets x 10reps
	Strength exercise C	2sets x 10reps
	Strength exercise B	2sets x 10reps
	Strength exercise F	2sets x 10reps
	Strength exercise D	2sets x 10reps
	Strength exercise H	2sets x 10reps
	Aerobic set 1	10 min
	Aerobic set 2	10 min
	Aerobic set 3	10 min
	Aerobic set 4	10 min
Cooling down		

EXERCISE TRAINING PROGRAM

The training program is divided into 5 phases of different duration (Table 2), starting with a familiarization phase of 4 weeks. All training phases after the familiarization period (i.e. phases 1, 2, 3 and 4) have the same structure. The first week of every phase was used to evaluate the strength training load (RM indirect measure) of the exercises used in this phase. Volume of aerobic training was reduced up to 120 minutes/week during this evaluation week. Selected intensities for strength training (50% RM and 70% RM) were applied from the second week on. Finally, the last week of each phase was used to learn the exercises used in the next phase and its proper technique and, consequently, the load was reduced. In every week (except for the evaluation one), participants performed 2 combined training sessions, and 1-2 aerobic sessions (depending on the selected frequency). Both combined sessions were different on the structure (so called session type A or B, see Table 3) or on the exercises performed. During the weeks of the same phase, participants repeated the same sessions every week.

In session type A strength straining aimed to maximize mechanical tensions and muscle damage, while in session type B strength training aimed to maximize metabolic stress. On session type A, the exercise order alternated between upper-body and lower-body, and agonist and antagonist exercises, allowing the maximum recovery possible to the implied muscle groups. On session type B, all upper-body exercises are performed before the lower-body exercises, and all agonist muscle groups exercises are also performed afterward each other.

Familiarization

This phase corresponds to the first four weeks of the training program. Participants performed a combined (aerobic plus strength) session 3 times per week, which means that during familiarization phase there was not “aerobic exclusive” training sessions. A sedentary person is not able to immediately train at the selected doses for ACTIBATE, therefore, the familiarization phase will allow participants to progressively increment the aerobic training volume until the selected dose is achieved (see Table 2). On the other hand, the strength training was based on light load training using elastic bands or weight bearing exercises, that increased in load and coordinative difficulty as soon as participants were able to perform the exercises with the proper technique. Strength training was focused on learning the main movement patterns to be used throughout the program (i.e. squat, hinge, bridge, lunge, planks, horizontal and vertical pulls, and horizontal and vertical push) and to improve: a) Core stability; b) Joint stabilizing muscles strength (e.g. rotator cuff, gluteus medius muscle, etc.); c) Balance and standing stability; and d) Flexibility. Strength training on the last week of the familiarization phase consisted on the same exercises performed in phase 1 (see below), but without considering the RM (i.e. light weights), so that participants learned the proper movement technique.

Phase 1

Aerobic training volume was 150 minutes/week (except for the evaluation week, see Table 2). The vigorous-intensity group exercised at 70% of HRres half of the aerobic training time (i.e. 60 minutes) in the 5th week of the training program. During the following weeks,

the vigorous-intensity group gradually increased the aerobic target intensity; thus, by the 7th week of the program, this group trained at the selected training intensity.

Strength training consisted on exercises localized in the main muscle groups (e.g. bench press, leg press, lat pull down, etc.) and included several compensatory exercises (e.g. core stability, stabilizers muscle, etc.) similar to those performed in the familiarization phase. Combined strength sessions (which includes aerobic and strength training) was a type A session (see Table 3) and included a total of 4 major muscle groups, 3 core stability and 4 compensatory exercises. Participants performed a total of 2 sets of 10 repetitions. For the vigorous-intensity group, strength training intensity was also gradually increased until the selected dose was achieved (see Table 2).

Phase 2

In the phase 2, combined sessions consisted on a type A session and a type B session (see Table 3). Strength training included similar exercises as those reported in the previous phase, as well as exercises involving several small muscle groups (e.g. arm curl, elbow extension, etc.), performing a total of 8-9 exercises per session. Both, session type A and session type B included the same strength exercises. Strength exercises were performed on 2 sets of 10 repetitions each session. On session type B, recovery time was 1 minute between sets and between different exercises.

Phase 3

Phase 3 include a type A session and a type B session (see Table 3). Both session A and session B contained the same strength exercises. Strength training included global exercises, which used simultaneously two or more kinetic chains (e.g. Lunge+horizontal press, hinge+horizontal pull, etc.) and, consequently, constitute a vigorous stimulus to stabilizers muscles such as core muscles. Strength exercises was performed on 2 sets of 10 repetitions each session. On session type B, recovery time was 1 minute between sets and between different exercises.

Phase 4

Phase 4 was divided into two periods, following an undulating periodization [21] that used similar stimulus than that used in phase 2 and 3, but emphasizing power training (i.e. maximum concentric speed, lower eccentric phase time, intra-set recoveries, etc.). The first 3-weeks period used the same sessions than phase 2 and the second 3-weeks period contained the phase 3 session types (see above).

Training sessions

The duration of each individual session depended on the number of visits per week (see above, frequency). Yet sessions were planned to evenly distribute the 150 minutes of aerobic PA throughout the week and not to prescribe aerobic training for longer periods than 60 minutes per session. There was a concern that longer periods of aerobic exercise would be an excessive burden and might have an adverse effect on adherence to the exercise intervention program due to a higher risk of injuries, fatigue and boredom. In order to distribute homogenously the training time across sessions, aerobic training time was distributed considering the strength training time, which means that combined training sessions (aerobic training and strength training) had a lower aerobic volume that the

aerobic ones. In every aerobic training session, participants alternated aerobic exercise with compensatory exercises (e.g. scapular and shoulder mobility and muscles activation, core stability, hip stabilizers muscle, balance exercises, etc.) that constitute a very low training load but might help to avoid boredom during sessions and prevent injuries.

Every combined training session started with a warm up consisting on 5 minutes of moderate intensity aerobic exercise (i.e. 60%HRres), and a set of mobility and activation exercises (e.g. front plank, monster walker, bridge, pelvic mobility, shoulder rotation, etc.). On the aerobic training sessions, exercisers only carried out the aerobic part of the warm-up. After warming-up, the aerobic training was performed on series of 10 minutes. Participants changed the ergometer (cycle ergometer, treadmill and elliptical ergometer) between series to avoid overuse of anatomical structures and boredom. The moderate intensity group kept the same intensity over time (60% HRres, SS exercise) whereas the intensity in the vigorous intensity group increased up to 80%HRres every 2.5 minutes in an interval manner (i.e. 60-80-60-80% HRres).

In the type A combined training sessions, strength exercises were intercalated between aerobic series, mainly to avoid monotony that could result in participants drop out. However, in type B sessions, all the strength training was performed before the aerobic training, and all aerobic series were performed one after the other. At the end of every session, participants performed a cooling-down protocol consistent on 2 minutes of aerobic exercise at a very light intensity and a set of stretching exercises of the main muscle groups involved in the training session.

ASSESSMENT PERIOD

After a thorough medical examination (anamnesis, blood pressure, resting electrocardiogram, etc.), the assessments period extended for 3 weeks, during which participants were due to came to the lab in 7 different occasions, in a randomized order (unless otherwise stated). This assessment period was conducted both before and after the training intervention program. Table 4 shows a summary of the different assessment carried out in every assessment day.

Table 4. Time distribution of the assessment period.

Assessment day	Measured groups of variables
Day 1	BIC, PPIT, SkTa, BCa, APP (ad libitum meal and appetite sensations)
Day 2	CVD (Blood samples)
Day 3	VO_2max
Day 4	MFOa
Day 5	STT
Day 6	PET-CT
Day 7	CIT, SkT
Day-living	ACC, Texpo
Several days	APP (24 hours dietary recalls), CVD (Blood pressure)

Groups of variables: a) PET-CT: Positron emission tomography-Computerized tomography; b) BIC: Basal indirect calorimetry; c) PPIC: Post-prandial indirect calorimetry; d) CEIC: Cold exposure indirect calorimetry; e) APP: Energy intake and appetite regulation; f) BCa: Body composition assessment; g) CVD: Cardiovascular disease risk factors; h) SkTa: Skin temperature assessment; i) MFOa: Maximal fat oxidation assessment; j) ACC: Accelerometry; k) Texpo: Outdoor and personal environmental temperature. VO2Max: maximum oxygen consumption; STT: Shivering threshold test.

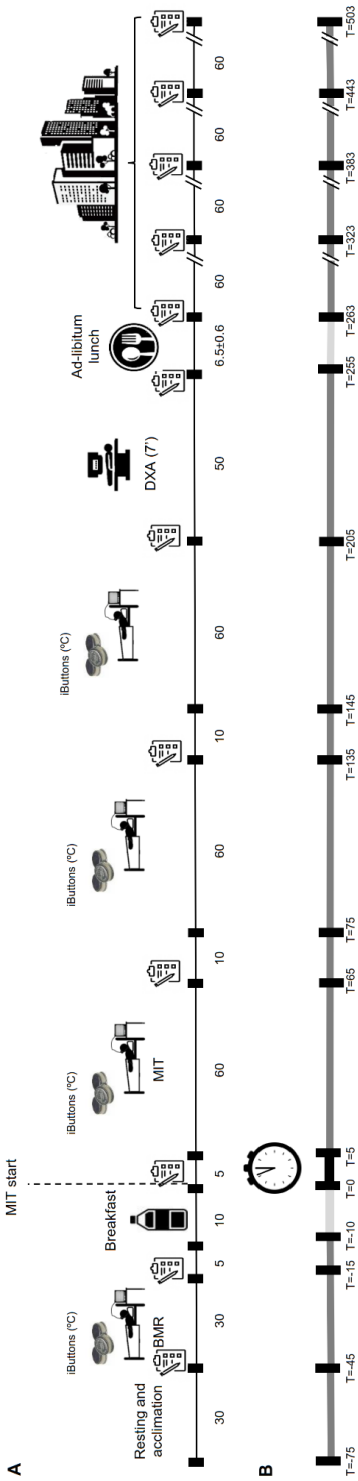

Figure 3. Day1 experimental procedure. A: General procedure and phases of the experimental protocol (numbers under the time lines represent minutes); B: Cumulative timeline (minutes). BMR: Basal metabolic rate; MIT: Meal induced thermogenesis; DXA: Dual-energy x-ray absorptiometry.

Day 1

For assessing basal and post-prandial EE and nutrient oxidation, body composition, and appetite regulation related variables, participants arrived at the lab early in the morning (8.15 am) after an overnight fasting (12 hours) and having consumed a standardized dinner (i.e. boiled rice, tomato sauce and egg omelet) the day before. They were also instructed to refrain from moderate or vigorous PA 24 and 48 hours respectively before the testing day, and to sleep as usual. All measurements were conducted in the same quiet and mild light room, with controlled ambient temperature (22-24°C) and humidity (35-45%). Figure 3 represent the experimental protocol of day 1.

After emptying their bladders, participants dressed in a short, flip-flops, and a standardized T-shirt (sandals, shorts and T-shirt, Clo=0.20). Immediately after, we measured the participant´s weight and height with a scale and stadiometer (Seca, model 799, Electronic Column Scale, Hamburg, Germany). Thereafter, participants lied in supine position on a horizontal bed for 15 minutes and then the bed was reclined for the rest of the measurements. Participants lied on the reclined bed at least 15 minutes before indirect calorimetry measures started. The same position and instructions were maintained during all measurements.

After resting, indirect calorimetry was recorded for 30 minutes, to assess the participant´s BMR. Immediately after the 30 minutes BMR measurement, participants were provided with a standardized liquid breakfast (T-Diet Energy neutral flavor, Vegenat S. A., Badajoz, Spain; http://vegenatnutricion.es/index.php?r=nutricion/producto&id=10) at 4°C, with an energy intake equivalent to 50% BMR (1.6 kcal/ml; 47% carbohydrates, 30% fat, 15% protein, 3% fiber). Participants had 10 minutes to take the breakfast and were allowed to drink water ad libitum.

Immediately after breakfast consumption, participants were instructed to lie on the reclined bed and were equipped with the gas collection system. Gas analyzers were recalibrated, and gas exchange recording started 5 minutes after the breakfast. Gas exchange was subsequently recorded during three 60 minutes periods, with a 10 minutes break (during which participants were allowed to get up from the bed but minimizing movement) between them (Figure 3). Gas analyzers were also recalibrated during every 10 minutes break. Of note, 3 hours and 25 minutes are representative of the total MIT response [22]. Skin temperature parameters were recorded concomitantly with indirect calorimetry measures by iButtons (DS-1922 L, Thermochron, resolution: 0.0625°C; Maxim, Dallas, USA).

Total urine production from the previous dinner to the breakfast, and from the standardized breakfast to the end of the MIT record were taken to correct EE and nutrient oxidation rates estimations.

After the MIT assessment, participants were moved into another room where a whole-body DXA scan (Discovery Wi, Hologic, Inc., Bedford, MA, USA) was performed to determine body composition. Finally, 4 h and 15 min after finishing the breakfast, participants were provided with an ad-libitum lunch. Appetite related sensations were assessed by VAS throughout the day (see figure 3).

Day 2

For blood sample collection, participants came early in the morning after an overnight fasting (12 hours) and avoiding PA for the 48 previous hours. A blood sample was taken from an antecubital vein, which was immediately centrifugated, aliquoted and stored at -80°C until analysed.

Day 3

For cardiorespiratory fitness assessment, participants came to the lab after a minimum of 4 hours fasting period. A maximum walking effort test in treadmill (H/P/cosmos pulsar, H/P/cosmos sports & medical GmbH, Nussdorf-Traunstein, Germany) was performed following the modified Balke protocol [23]. Gas exchange was continuously recorded with a CPX UltimaCardiO2 metabolic cart (Medgraphics Cardiorespiratory Diagnostic, Saint-Paul, USA).

Day 4

For assessing MFO and Fatmax, after a fasting period of 5-6 hours, the participants went through a submaximal walking effort test in a treadmill (H/P/cosmos pulsar, H/P/cosmos sports & medical GmbH, Nussdorf-Traunstein, Germany). The protocol began with a maximal walking speed test (selecting the individual maximum comfortable walking speed between 5.5, 6.5, and 7.5 km/h). Then, the graded exercise protocol started with 3 minutes warm-up at 3.5 km/h (gradient 0%), and the speed was increased by 1 km/h every 3 minutes until the maximal walking speed was reached. Thereafter, the treadmill speed was kept constant (i.e. maximal walking speed) with the gradient increasing by 2% every 3 minutes until the RER was higher than 1.0 [24]. Respiratory gas exchange was also registered during the whole test.

Day 5

For assessing the individual shivering threshold participants came to the lab after a minimum of 6 hours fasting. They were asked to come by bus or by car, to sleep as usual, to refrain from any moderate (in the previous 24 hours) or vigorous (in the previous 48 hours) PA, and not consume alcoholic or stimulant beverages over the past for 6 hours. The participants were evaluated between 8.30 and 19.15hrs. After having checked that they met the previous conditions, the participants were equipped with the same standardized clothes that for the basal and post-prandial EE assessment. Later they rested in a warm room (22-23°C) for 30 minutes. Later, they were entered into a mild cold room (19.5-20°C) and dressed in a water perfused vest (Polar Products Inc., Ohio, USA) set at ≈17°C. Thereafter, the water temperature was slightly decreased (approximately 2°C every 10 minutes) [25] until a temperature of 3.8°C was reached (at which the participants remained exposed for 45 additional minutes) or shivering occurred. We determined shivering visually and by asking the participants if they were experiencing shivering. The water temperature at which shivering occurred was considered the shivering threshold (i.e. the lowest tolerable temperature without external observation or auto-reporting shivering) [25].

Day 6

Forty-eight or seventy-two hours after assessing the shivering threshold test, participants came to the lab after following exactly the same instructions to assess their BAT and skeletal muscle ^{18}F-FDG activity after a personalized cold stimulation. They were again equipped with the standardized clothes and were required to rest during 30 minutes in a warm room. Later, they were moved to the mild cold room (19.5-20°C) and equipped with the water perfused cooling vest (Polar Products Inc., Ohio, USA) set at a temperature 4°C higher than the individual shivering threshold. Water temperature was increased by 1°C if the participants started to shiver. Participants remained seated and cold exposed for 2 hours. After the first hour of cold exposure, an intravenous bolus (≈185 MBq) of ^{18}F-FDG was injected through an antecubital catheter, and water temperature was increased 1°C to avoid shivering. At the end of the two hours cold exposure, participants went through a static PET-CT scan (Siemens Biograph 16 PET-CT, Siemens, Germany). The PET-CT images were taken from the atlas vertebrae (Cervical 1) to the thoracic vertebrae 6 (2 BEDs), approximately. 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.

Day 7

Finally, 48-72 hours later than the PET-CT, participants came to the lab again following the same previous instructions than in days 5 and 6, to assess CIT and CI-NUTox. After resting in the reclined bed for 20 minutes, participant´s RMR was measured for 30 minutes in the warm room (22-23°C). Later, the participants were moved into the cold room (19.7±0.4°C) and dressed in a temperature-controlled water perfused cooling vest (Polar Products Inc., Ohio, USA) set 4°C above the individual's shivering threshold temperature. They then lay down on a bed with the same reclined position as the one used for the RMR assessment. Again, they were instructed to breathe normally, and not to talk, fidget, or sleep. Then, indirect calorimetry measurement was performed during two consecutive 30-minute periods, separated by a 5-minute pause to recalibrate the metabolic cart.

OUTCOME MEASURES

Positron emission tomography-Computerized tomography (PET-CT)

We analyzed the PET-CT images using the Beth Israel plugin for FIJI software [26]. Following current recommendations for BAT assessment [27], we applied a fixed range of (−190 to −10 HU). We calculated BAT volume, BAT metabolic activity, BAT mean activity (SUV mean), and BAT maximal activity (SUV peak). The region of interest was semi-automatically outlined from the atlas vertebrae (Cervical 1) to the thoracic vertebrae 4. To obtain BAT volume, BAT metabolic activity and BAT SUV mean we used two different SUV thresholds: i) Individualized SUV threshold [1.2/(lean body mass/body mass)] [27]; and ii) Fixed SUV threshold (SUV=2) [2]. BAT mean radiodensity (HU), was also obtained from the software, applying the individualized SUV threshold. Moreover, BAT SUVmean and SUVpeak were expressed either as a function of body mass (SUV_{BM}) and as a function of lean body mass (SUV_{LBM}).

In addition, we calculated the SUVpeak of several skeletal muscles (paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii), and averaged the obtained value from all muscles in both sides of the body. Furthermore, we grouped these muscles into deep (paracervical, scalene, longus colli, paravertebral, subscapular), cervical (paracervical, sternocleidomastoid, scalene, longus colli), and cold sensitive (sternocleidomastoid, scalene, longus colli, pectoralis major) muscles [28]. We also computed the SUVpeak of the descending aorta, tricipital subcutaneous white adipose tissue, and back subcutaneous white adipose tissue (behind thoracic vertebrae 2), expressing them as SUV_{BM} and SUV_{LBM}.

Basal indirect calorimetry (BIC)

For assessing BMR, indirect calorimetry measurements were performed with one of two different metabolic carts: CCM and MGU (Medgraphics Corp, Minnesota, USA), using either a ventilated face-tent system or a neoprene face-mask without external ventilation [29]. Flow calibration was performed by a 3-L calibration syringe at the beginning of every testing day, and gas analyzers were calibrated using 2 standard gas concentrations following the manufacturer's instruction before every indirect calorimetry measurement. Participants were instructed to breath normally, and not to talk, fidget or sleep.

Data treatment for BMR was tested and selected in the methodological study 1 (see below). To determine BMR, data were averaged every minute and downloaded from the Breeze Suite (8.1.0.54 SP7) software. The first 5 minutes were deleted and the CV of VO_2, carbon dioxide production VCO_2, RER and VE were calculated for every 5 minutes period (i.e. from minute 6 to 10; from minute 7 to 11, etc.). Later, we selected those periods fulfilling the highest number of SS criteria: i) CV <10% for VO_2, ii) CV <10% for VCO_2, iii) CV <10% for VE, and iv) CV <5% for RQ. Finally, among the periods meeting the highest number of criteria, we selected the one with the lowest average among the CVs of VO_2, VCO_2, VE and RER. Mean VO_2 and VCO_2 of this 5-minute period were considered for further analysis [30]. Participants presenting a basal RER <0.7 or >1 (n=3; 2 men) were excluded from the analyses.

We estimated EE and the nutrient oxidation rates using standard stoichiometry equations proposed by Weir (a) [31] and Frayn (b,c) [32].

(a) $EE\ (\text{kcal/min}) = 3.941 \times VO_2\ (\text{l/min}) + 1.106 \times VCO_2\ (\text{l/min}) - 2.17 \times \text{N(g/min)}$

(b) $CHOox\ (\text{g/min}) = -3.21 \times VO_2(\text{l/min}) + 4.55 \times VCO_2(\text{l/min}) - 2.87 \times \text{N(g/min)}$

(c) $FATox\ (\text{g/min}) = 1.67 \times VO_2(\text{l/min}) - 1.67 \times VCO_2(\text{l/min}) - 1.92 \times \text{N(g/min)}$

For logistic reasons, we could not assess nitrogen in all urine samples. Therefore, we build a predictive equation form a linear regression model using data of urine urea concentration (Spinreact, Catalogue No. 283-17) and nitrogen concentration (Kjeldahl method) from some of the participants (n=19, 16 women; 21.87±2.05 years old; 24.87±3.71 kg/m^2). Then, we measured total urine volume and urea concentration (Spinreact, Catalogue No. 283-17) in both urine samples (for BMR and MIT) of every participant. Thereafter, we estimated nitrogen urine levels from urea concentration following the equation "Nitrogen (g/l) = 0.0065* Urea (mg/dl) + 1.2598" obtained from the linear regression

(R^2=0.851; Figure 4). When the urea concentration was missing we assigned a value of 0.0083 g/min for nitrogen excretion [33].

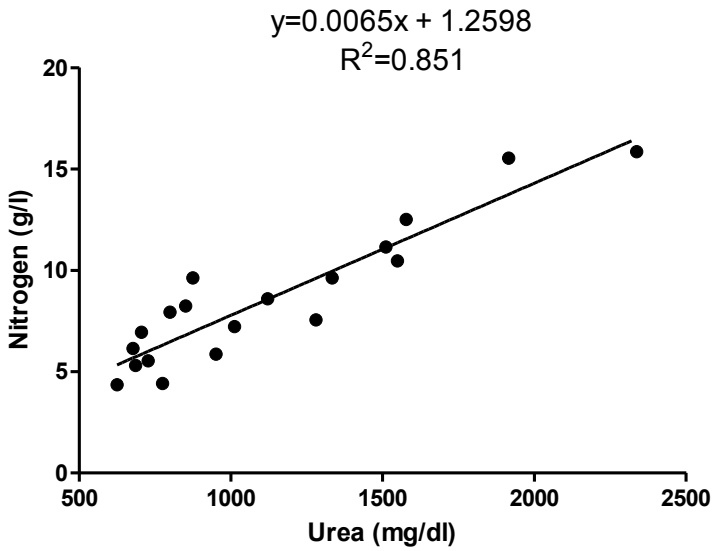

Figure 4. Association between urine urea and nitrogen concentrations in 19 adults (n=19, 16 women; 21.87±2.05 years old; 24.87±3.71 kg/m^2).

Post-prandial indirect calorimetry (PPIC)

For assessing MIT, the same metabolic cart (equipped with the same gas collection system) used for the BMR assessment was employed. Immediately after breakfast consumption, participants were instructed to lie on the reclined bed and were equipped with the gas collection system. Gas analyzers were recalibrated, and gas exchange recording started 5 minutes after the breakfast. Gas exchange was subsequently recorded during three 60 minutes periods, with a 10 minutes break (during which participants were allowed to get up from the bed but minimizing movement) between them (Figure 3). Gas analyzers were also recalibrated during every 10 minutes break. Of note, 3 hours and 25 minutes are representative of the total MIT response [22].

To determine MIT, the first 10 minutes of each 60 minutes recording periods were excluded. Subsequently, every 60 minutes period was divided into five 10 minutes periods from which the most stable 5 minutes period (following the same method described for BMR calculations) was selected and averaged. Later, we calculated the AUC following the trapezoidal rule and expressed it as a percentage of the individual´s energy intake. We also calculated several deltas to assess metabolic flexibility: a) The difference between the maximum RER value during the post-prandial period and the one from the BMR assessment; b) The difference between the minimum RER value during the post-prandial period and the one from the BMR assessment. The same deltas were also calculated for CHOox and fat FATox oxidation. We also calculated the percentage of BMR attributable to FATox.

As in BMR (see above), we estimated EE and the nutrient oxidation rates using standard stoichiometry equations proposed by Weir (a) [31] and Frayn (b,c) [32].

Cold exposure indirect calorimetry (CEIC)

The indirect calorimetry measurements in day 7 (for both RMR and CIT) were performed using a neoprene face-mask hooked up to a CCM/ MGU device equipped with a directconnect™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA) [29,30]. Flow calibration was performed by a 3-L calibration syringe at the beginning of every test day while gas analyzers were calibrated using 2 standard gas concentrations before every bout of 30 minutes indirect calorimetry measurement. We used the same metabolic cart for RMR and CIT in all participants.

Data treatment for CIT was tested and selected in the methodological study 2 (see below). Indirect calorimetry data were averaged every minute and downloaded from the Breeze Suite (8.1.0.54 SP7) software. For RMR, we selected the most stable 5-minute period (i.e. the one with the lowest average of VO_2, VCO_2, VE and RER, CVs), after excluding the first 5 minutes recorded [30]. For CIT, we also excluded the first 5 minutes record, and averaged every 5 minutes (from 6th to 10th, from 11th to 15th, etc.).

Oxygen consumption and carbon dioxide production for each selected data point were used to estimate EE, and CHOox and FATox. EE was estimated through Weir´s abbreviated equation, not considering urinary nitrogen concentration (a) [31]. For CHOox (b) and FATox (c) estimations, we used Frayn´s equation, not considering urinary nitrogen concentration [32].

(a) $EE\ (\text{Kcal/min}) = 3.941 \times VO_2\ (\text{l/min}) + 1.106 \times VCO_2\ (\text{l/min})$

(b) $CHOox\ (\text{g/min}) = -3.21 \times VO_2\ (\text{l/min}) + 4.55 \times VCO_2\ (\text{l/min})$

(c) $FATox\ (\text{g/min}) = 1.67 \times VO_2\ (\text{l/min}) - 1.67 \times VCO_2\ (\text{l/min})$

To obtain a single representative value of CIT, we divided the 60 minutes recorded into 4 periods (i.e. 15 minutes each). We then selected the most stable 5-minutes period within every 15-minutes period. Finally, we used the 4 selected 5-minutes periods together with the RMR, to calculate the AUC (trapezoidal rule) expressed as a percentage of RMR.

Despite our careful assessment of the shivering threshold, visually detected and auto-reported shivering was recorded in 17 (16 women), and those individuals were therefore excluded from further analysis. In addition, participants (2 men) presenting RER values higher than 1.1 or lower than 0.7 at any measure point, or a RER higher than 1.0 in RMR assessment, were also excluded from the analysis [34]. For nutrient oxidation rates analysis, only the participants with a fasting time between 6 and 8 hours were considered [24,35].

Energy intake and appetite regulation (APP)

For the ad-libitum meal, participants were offered a plate of spaghetti (i.e. spaghetti, tomato sauce, pork tenderloin and virgin olive oil), prepared in the center immediately before. The lunch composition was 45.5% carbohydrates, 38.5% fat, and 16% proteins of the total energy, with an energy density of 1.54 kcal/g. The total amount offered was 1500 g for men and 1000 g for women. Participants were then ushered in to a quiet, dim lit room where they found the plate together with a glass with 450 ml of water, were alone and without external distractors while eating. The participants were instructed to eat until comfortably satisfied. Food intake was measured by differences in spaghetti weight before and after lunch, and the energy intake was subsequently calculated.

Habitual energy intake was estimated by means of three non-consecutive 24-h dietary recalls (one of them for a non-working day). Thereafter, all the foods and drinks consumed on the previous day were recorded. A book with pictures of different food servings and sizes was used to help the participants to estimate the amount of food consumed. Nutritional composition of the diet was obtained by the EvalFINUT software (http://www.finut.org/evalfinut/). The consumption of water and salt was not collected. The participants were not informed when they were going to be evaluated so as not to bias the data. Participants were classified as plausible or non-plausible reporters using the Goldberg's cut-off method [36]. The PAL was obtained from an accelerometer (see below). We assigned a PAL of 1.8 to the participant with the highest activity level, and a PAL of 1.4 to the participant with the lowest activity level. For the rest of participants, we calculated the proportional value between 1.4 and 1.8.

During day 1, participants' subjective appetite sensations (i.e. hunger and fullness) were recorded with VAS, using pen and paper, at several time points. Participants were asked to complete the VAS on arriving; before the BMR assessment; immediately before the breakfast; immediately after, and 65, 135 and 205 min after the breakfast; immediately before the ad-libitum lunch; immediately after, and 1, 2, 3 and 4 h after the lunch (see figure 3). Every VAS was completed in the lab, except for those after the lunch, which was completed in free-living conditions (participants were instructed not to eat during this period). VAS as written in Spanish had been validated in a previous study [37].

Body Composition assessment (BCa)

A DXA scanner (Discovery Wi, Hologic, Inc., Bedford, MA, USA) was used to measure BMD (g/cm^2). The whole-body scan was used to obtain BMD of the total body and the lumbar spine (mean of L1-L4). We performed the quality controls, the positioning of the participants, and the analyses of the results following the manufacturer's recommendations. An automatic delineation of the anatomic regions was performed by the software (APEX 4.0.2). We acquired spine phantom quality-control scans on each study day. In addition, fat mass, lean mass and visceral adipose tissue mass were obtained from the DXA scan. Body, lean and fat mass indexes were calculated as mass (kg) / Height $(m)^2$. We also calculated body surface area following the DuBois and DuBois formula [Body surface area = $(\text{Weight (Kg)}^{0.425} \times (\text{Height (cm)}^{0.725}) \times 0.007184$] [38].

Cardiovascular disease risk assessment (CVD)

Serum glucose determination was performed in a Beckman Coulter AU5832 analyzer using the reactive OSR6521 (Beckman Coulter Inc., CA, USA). Insulin concentration was determined by chemiluminescent immunoassay in a DXI analyzer (Beckman Coulter Inc., CA, USA). Serum triglycerides, total cholesterol and HDL concentrations were determined by colorimetric methods in the Beckman Coulter AU5832 analyzer using the reactive OSR61118, OSR6516 and OSR6587 (Beckman Coulter Inc., CA, USA), respectively. LDL concentration was later calculated using the Friedewald equation [39]. Insulin resistance was estimated by means of the HOMA: fasting insulin (mU/L) x fasting glucose (mmol/L) / 22.5 [40].

Blood pressure was measured in three different days (initial medical examination, day 5 and day 6) with an automatic sphygmomanometer Omrom M2 (Omron Healthcare, Kyoto, Japan) and an average was calculated.

Skin temperature assessment (SkTa)

As stated above, skin temperature was recoded concomitantly to the BMR, MIT and CIT assessments. Seventeen iButtons (DS-1922 L, Thermochron, resolution: 0.0625°C; Maxim, Dallas, USA) were attached to participant's skin with adhesive tape (Fixomull, Beiersdorf AG, Hamburg, Germany) on several body regions [41]. iButtons are valid and reliable devices to measure skin temperature in humans [42]. The iButtons registered temperature at a frequency of 1Hz. Mean, proximal and distal skin temperatures parameters were calculated following previously published equations [43–46]. Of note, supraclavicular skin temperature was assessed directly by the iButton placed in the supraclavicular fossae. All data registered by the devices and the gradient were analyzed by the Temperatus software (http://profith.ugr.es/temperatus). Averages of every 5 minutes period were obtained for further analyses. We calculated the AUC (trapezoidal rule) to compute the meal-induced increases in skin temperature as a single value, and then calculated the difference between this AUC and the BMR measurement.

Maximal fat oxidation assessment (MFOa)

For exercise indirect calorimetry record (i.e. MFO and maximum effort test) data were averaged every 10 seconds and downloaded from the Breeze Suite (8.1.0.54 SP7) software. In the maximum effort test, only the value with the highest oxygen uptake (after discarding occasional artefacts) was used in further analyses (to determine Fatmax). In MFO test, gas exchange data of the last 60 seconds of each intensity stage were averaged. Later, we calculated MFO and Fatmax constructing a third polynomial curve with intersection at (0;0) from a graphical depiction of FATox values as a function of exercise intensity [47]. As in CIT, CHOox and FATox were estimated from oxygen consumption and carbon dioxide production using Frayn´s equation, not considering urinary nitrogen concentration [32].

Accelerometry (ACC)

PA was objectively measured with a wrist and a hip-worn accelerometer (ActiGraph GT3X+, Pensacola, FL, US) for 7 consecutive days (24 hours) [48,49]. The accelerometers were initialized to store raw accelerations at a sampling frequency of 100 Hz. The raw accelerations were then processed in ActiLife v. 6.13.3 software (ActiGraph, Pensacola, FL, US) to obtain activity counts from the vector magnitude over 5-second epochs using the default filter of ActiLife. The participants who wore the accelerometers for ≥ 10 hours/day and 4 hours/night during at least 4 days were included in the analyses [48]. We used the mean counts per 5 seconds over the entire day (i.e., 24 hours) as an indicator of the PA level [49].

Thermal exposure (Texpo)

Participants wore 1 iButton (DS-1922 L, Thermochron; resolution: 0.0625 °C; frequency: 10 min intervals; Maxim, Dallas, USA) for 7 days. The iButton was attached to a plastic fob and was used to quantify the Personal-ET. The iButton remained with the participant all the time but was never in direct contact with the body or under clothing. During sleep-phases, the iButton was placed on the bedside table. The iButton was 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 wearing days.

STUDIES' METHODOLOGY OVERVIEW

The present doctoral thesis contains a total of 10 studies. Two of them (methodological study 1 and 2) were designed to determine methodological issues related with BMR and CIT data analysis. The rest of them (studies 3-10) were conducted to address the aims of the doctoral thesis (see aims section). All studies, except the methodological study 1 contains data from the participants enrolled in the ACTIBATE study. The independent cohort used in study 1 will be referred as "BMR-reliability". On the other hand, study 1 and study 2 contain also data from other cohorts different than the ACTIBATE study (referred as "CIT-confirmatory"). Table 5 shows an overview of the design, cohorts, and variables included in every study contained in this doctoral thesis.

Table 5. Studies' methodology overview.

Study	General aim	Design	Cohort and participants	Study outcomes
Methodological study 1	To compare different BMR data treatments	Repeated measures	BMR-reliability (n=17)	BIC
Methodological study 2	To compare different CIT data treatments	Repeated measures	ACTIBATE (n=44)	CEIC
Study 3	To study the effect of cold exposure on CIT	Repeated measures	ACTIBATE (n=44), and CIT-confirmatory (n=13)	CEIC
Study 4	To study the association of BAT and skeletal muscle ^{18}F-FDG act. with CIT	Cross-sectional	ACTIBATE (n=44), and CIT-confirmatory (n=13)	PET-CT, CEIC, SkTa
Study 5	To study the association of BAT and skeletal muscle ^{18}F-FDG act. with BMR and MIT	Cross-sectional	ACTIBATE (n=112)	PET-CT, BIC, PPIC, SkTa
Study 6	To study the association of BAT and skeletal muscle ^{18}F-FDG act. with APP	Cross-sectional	ACTIBATE (n=144)	PET-CT, APP, BCa
Study 7	To study the association of BAT with bone mineral density	Cross-sectional	ACTIBATE (n=98)	PET-CT, BCa, ACC, Texpo
Study 8	To study the association of BAT with BC (fat and lean mass)	Cross-sectional	ACTIBATE (n=114)	PET-CT, BCa, CVD
Study 9	To compare BAT and skeletal muscle ^{18}F-FDG act. and energy balance related variables between MHOO and MUOO	Case-control	ACTIBATE (n=53)	CVD, BCa, PET-CT, BIC PPIC, CEIC, APP, MFOa
Study 10	To study the effect of the exercise program on BAT and skeletal muscle ^{18}F-FDG act., and energy balance related variables	Randomized controlled trial	ACTIBATE (n=82)	PET-CT, BIC, PPIC, CEIC, APP, BCa

Groups of variables: a) PET-CT: Positron emission tomography-Computerized tomography; b) BIC: Basal indirect calorimetry; c) PPIC: Post-prandial indirect calorimetry; d) CEIC: Cold exposure indirect calorimetry; e) APP: Energy intake and appetite regulation; f) BCa: Body composition assessment; g) CVD: Cardiovascular disease risk factors; h) SkTa: Skin temperature assessment; i) MFOa: Maximal fat oxidation assessment; j) ACC: Accelerometry; k) Texpo: Outdoor and personal environmental temperature. BAT: Brown adipose tissue; 18F-FDG: Fluorodeoxyglucose; MHOO: metabolically healthy overweight or obese; MUOO: Metabolically unhealthy over-weight or obese.

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Reliability of resting metabolic rate measurements in young adults: Impact of methods for data analysis

STUDY 1

BACKGROUND

Measuring human RMR is of key relevance in research and in the clinical setting [1–3]. Among the available methods to measure RMR, IC through a metabolic cart is the most commonly used in healthy, non-critically ill and ventilated individuals. In IC, EE is calculated from measured VO_2 and VCO_2 by using estimating equations [4,5]. Additionally, nutrient oxidation rates (i.e. CHOox and FATox) can be estimated from IC measurements [6]. Guidelines on how to perform IC evaluations were published more than a decade ago [7] and were recently updated [8]; yet, there are still some issues that need to be clarified [8].

When performing IC with metabolic carts, gas exchange is commonly recorded during a relatively short period of time (e.g. 30 minutes), from which a shorter period of recorded data is selected and analyzed (e.g. 5 minutes). It is assumed that the selection of a SS period, defined as a period in which gas exchange variables present low variation, increases the validity of the measure [9]. SS is commonly established as a period during which average minute VO_2, VCO_2, RER, and/or VE CVs is lower than a pre-determined percentage (usually 10% for VO_2, VCO_2, and VE, and 5% for RER) [9]. However, as SS is not always feasible to achieve, other methods for data analysis have been proposed [10].

Methods for data analysis can be grouped in those based on a pre-defined TI selection and those based on SSt approach [10]. Of note is that there is no consensus about time length of data selection in both TI or SSt methods [8,10,11], neither about the selection of which gas exchange variables and which pre-defined CV is better for determining SS [8]. High inter-day reliability is a key factor to analyze the magnitude of change in RMR after an intervention [12,13]. Moreover, although RMR estimation is mainly dependent on VO_2 [4], the ratio between VO_2 and VCO_2 (i.e. RER) is crucial for estimating nutrient oxidation rates [5,14]. Consequently, achieving a high RER reliability is also key for a method to be able to accurately estimate fuel oxidation. However, to our knowledge there are no studies examining the impact of different methods for data analysis (i.e. TI and SSt) on inter-day RMR and RER reliability.

The assumption that SS provides more valid RMR and RER measurements comes mainly from studies performed with ventilated patients [9,15]. However, it is unknown whether this also applies to healthy non-ventilated people [15,16]. On the other hand, it has been shown that RMR is consistently lower when following SSt than when following TI methods in healthy individuals achieving SS [10]. This suggests that achieving SS could provide a more valid RMR measure, given that RMR is considered the lowest EE in an awake person [10]. However, whether the inter-day RMR or RER reliability is higher in individuals achieving the SS compared to those that do not achieve the SS needs to be studied.

The aims of this study were: i) to analyze the impact of methods for data analysis (TI and SSt) on RMR and RER measurements in young adults; ii) to analyze the impact of methods for data analysis (TI and SSt) on inter-day RMR and RER reliability; iii) to compare inter-day RMR and RER reliability across methods for data analysis (TI and SSt) in participants who achieved SS vs. participants who did not achieve SS.

METHODS

Participants

A total of 20 (n=13 women) Caucasian young healthy adults aged 18-26 years participated in the study. A total of 3 out of 20 participants did not meet the previous conditions for IC measurements on one of the testing days (2 participants performed PA in the 24 hours prior to the measurement, and the other one did not meet the minimum fasting time requirement). Consequently, they were retrospectively excluded from further statistical analyses. They were non-physically active (<20 minutes <3 days/week), had a stable body weight (body weight changes <3 kg) over the last 3 months, were not enrolled in a weight loss program, were non-smokers, did not take any medication, had no acute or chronic illness, and were not pregnant. The study protocol and informed consent were performed in accordance with the Declaration of Helsinki (revision of 2013) and 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). Written informed consent was obtained from all the participants before their enrollment. Table 6 shows the methodology overview.

Table 6. Study 1 methodology.

GENERAL INFORMATION	
General aim	To compare different BMR data treatments
Design	Repeated measures
Cohort and participants	BMR-reliability (n=17)
OUTCOMES INCLUDED IN THE STUDY	
Outcome	Variables included
BIC	BMR RER

BIC: Basal indirect calorimetry; BMR: Basal metabolic rate; RER: Respiratory exchange ratio.

Procedures

The study was conducted between February and April 2016. IC was measured via a repeated-measures design over 2 consecutive days. Measurements were conducted between 7.30 AM and 11 AM, and each participant was given an appointment at the same time on both days. Participants arrived at the laboratory by car or by bus (avoiding any PA after waking up) in a fasted state (at least 8 hours). They were instructed to refrain from moderate or vigorous PA 24 and 48 hours before the testing day, respectively. On each testing day, before performing the measurements, participants had to confirm that they met the aforementioned study conditions.

On both testing days, IC measurements were performed during two consecutive 30-minute periods with two different metabolic carts: CCM and MGU (Medgraphics Corp, Minnesota, USA), using neoprene face-mask without external ventilation. The device order was replicated on both testing days, and it was counterbalanced between participants. Both devices measure VO_2 and VCO_2 using a breath-by-breath technique for

determining the gas exchange. VCO_2 measurement is performed using a non-dispersive infrared analyzer, and VO_2 is measured using a galvanic fuel cell [17,18].

IC measurements followed current guidelines [8]. In brief, all measurements were conducted in the same quiet room with dim lighting, with controlled ambient temperature (22-24°C) and humidity (35-45%), and by the same trained staff. Before being evaluated, all participants confirmed that they met previous study conditions and lied on a reclined bed in a supine position and covered by a sheet for the 20 minutes prior to the IC measurement. They were instructed to breathe normally, and not to talk, fidget, or sleep. The same position and instructions were maintained during the two 30-minute measurement periods. Flow calibration was performed by using a 3-L calibration syringe at the beginning of every testing day, and gas analyzers were calibrated using 2 standard gas concentrations following the manufacturer's instruction before every IC measurement.

On day 1, we measured participants' weight and height using a Seca scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Participants wore light clothing and no shoes during the measurements.

Methods for data analysis and steady state criteria

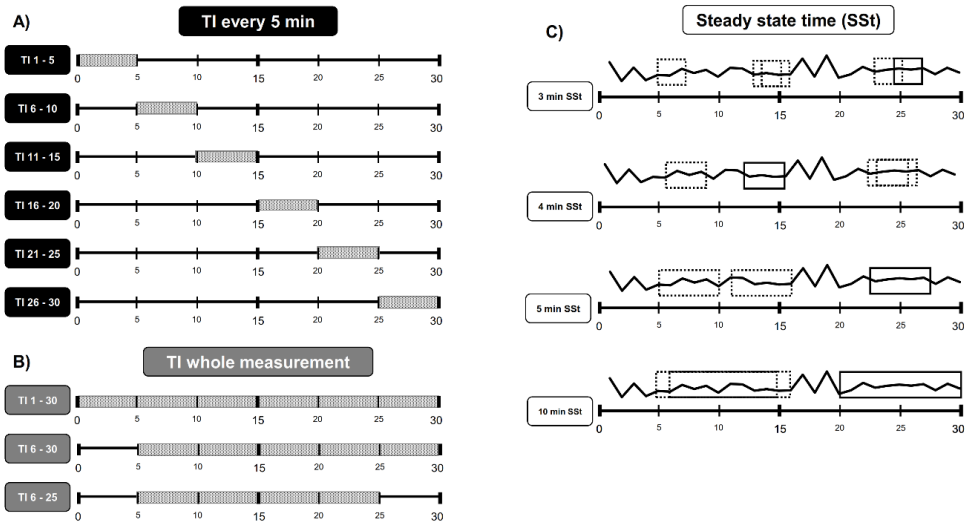

Figure 5. Methods for data analysis. A=Time interval (TI) methods every 5 minutes; B= TI for the whole measurement. Pointed blocks represent selected data for each method for data analysis in A and B panels; C= Steady state time (SSt) methods. Y axe represents resting metabolic rate (simulated data). Blocks represent the 3, 4, 5, or 10-minute periods that met most of the following criteria: CV<10% for VO2, VCO2, and VE, and CV<5% for RER, among the 30-minute record, after having discarded the first 5 minutes recorded. The solid lined blocks represent the period with the lowest average between CVs of VO2, VCO2, VE, and RER, and thus, the period of time selected in each method for data analysis. Dashed lined blocks represent periods with the same number of CVs criteria achieved as the selected period but with a higher average between CVs of VO2, VCO2, VE, and RER.

We used two types of methods for data analysis based on TI and SSt periods. (Figure 5): (i) TI every 5 minutes, and TI representing the whole measurement period; and (ii) SSt methods. TI every 5 minutes: mean values of every consecutive 5-minute period (i.e. from the 1st to the 5th minute, from the 6th to the 10th, etc.), hereinafter referred as *1-5 min*, *6-10*

min, *11-15 min*, *16-20 min*, *21-25 min*, and *26-30 min* (Figure 5A). TI representing the whole measurement period: mean values for the whole measurement period (i.e. *1-30 min*), and mean values for the whole measurement period except for the first 5 minutes (i.e. *6-30 min*) [8] or the first and the last 5 minutes (i.e. *6-25 min*) [19] (Figure 5B). For the SSt methods, we calculated the CV of VO_2, VCO_2, VE, and RER for every period of 3, 4, 5, and 10 minutes [7, 11], excluding the first 5 minutes of data collection (i.e. for *3 min SSt*, CVs were calculated from 6th to 8th minute, from 7th to 9th, etc.) (Figure 5C). Thereafter, we selected the periods of 3, 4, 5, or 10 minutes that met most of the following criteria: i) CV<10% for VO_2, ii) CV<10% for VCO_2, iii) CV<10% for VE, and iv) CV<5% for RER. Finally, among the periods that met most of those criteria we selected the 3, 4, 5, and 10-minute periods with the lowest average between CVs of VO_2, VCO_2, VE, and RER, for being used as *3 min SSt*, *4 min SSt*, *5 min SSt* and *10 min SSt*, respectively (Figure 5C). Finally, mean VO_2 and VCO_2 obtained by each method for data analysis were entered into Weir´s abbreviated equation [4] (see below) to estimate EE, and RER was calculated as VCO_2/VO_2:

$$RMR\ (\text{Kcal/min}) = 3.941 \times VO_2(\text{l/min}) + 1.106 \times VCO_2(\text{l/min})$$

To compare inter-day RMR and RER reliability across methods for data analysis in participants who achieved SS vs. participants who did not achieve SS, we classified participants as those achieving CV<10% for VO_2, VCO_2 and VE and CV<5% for RER (SS criteria) and those who failed to comply the SS criteria on any of the two testing days. This classification was performed for every method for data analysis. Therefore, a total of 13 methods for data analysis were tested, and were further grouped in those achieving SS vs. not achieving SS.

The selected CV cut-off points are probably the most used ones in literature [8]. In addition, a CV cut-off point of 10% for VO_2 and VCO_2 has been proved to accurately predict total EE in ventilated patients [9]. However, there is no consensus on how to define SS, neither on CV cut-off points, nor in the combination of gas exchange variables. Therefore, we selected the most used CV cut-off points [8], and we decided to classify participants taking into account the four gas exchange variables. This is the strictest combination criteria, which would allow to test whether achieving SS would result in better inter-day reliability. Nevertheless, we performed additional analyses classifying participants just based on VO_2 and VCO_2 CV criteria.

Statistical analysis

Gas exchange parameters including VO_2, VCO_2, VE, RMR, and RER were averaged each minute with the Breeze Suite (8.1.0.54 SP7, MGCDiagnostic®) software and downloaded to an Excel spreadsheet where the CVs and outputs of the different methods for data analysis were calculated. Results are presented as means ± standard deviation, unless otherwise stated. The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 21.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set to <0.05.

Impact of methods for data analysis on RMR and RER measurements

A repeated-measures ANOVA was used to test differences in RMR and RER measurements across methods for data analysis for both CCM and MGU metabolic carts

on both testing days. LSD Tuckey and Bonferroni corrections were used to perform post hoc comparisons.

Impact of methods for data analysis on inter-day reliability

We compared the absolute value of inter-day differences in RMR and RER values (e.g. |RMR Day1 – RMR Day2|) with every method for data analysis using repeated-measures ANOVA for both CCM and MGU metabolic carts. Inter-day RMR and RER reliability for every method of data analysis was also assessed using the Bland-Altman method [20]. Day 1 measurements were subtracted from day 2 measurements, so a positive difference indicates that day 2 measurements were higher than day 1. Bias was measured by using a 2-sided *t*-test to determine if there was a significant difference between RMR and RER measures on day 2 vs. day 1.

Inter-day reliability across methods for data analysis in participants achieving SS vs. not achieving SS

The absolute value of inter-day differences in RMR and RER (e.g. |RMR Day1 – RMR Day2|) in each method for data analysis and for both CCM and MGU metabolic carts were compared between those participants who achieved the SS criteria and those who did not, using independent sample *t*-tests.

RESULTS

The included participants (n=17, 11 women) were 23.2±1.9 years old. Mean weight and height were 63.3±11.5 Kg and 168±9 cm respectively (BMI: 18.6 to 26.2 kg/m^2). All participants had valid data for the MGU, and all except one had valid data for the CCM (n=16).

Impact of methods for data analysis on RMR and RER measurements

Figure 6 shows mean values of day 1 measurements for RMR and RER across different methods for data analysis. Repeated-measures ANOVA indicated significant differences in mean RMR and RER for both CCM and MGU metabolic carts (all P<0.01). The lowest RMR value was obtained when following the *5 min SSt* method for both CCM and MGU. The lowest RER value was also obtained following the *5 min SSt* for the CCM, but not for the MGU, where *1-5 min* method resulted in lower RER value. LSD Tuckey post hoc comparisons revealed significant differences between SSt and TI methods. SSt obtained lower values, but we found no significant differences when comparing between different lengths of SSt methods. Nevertheless, significant differences disappeared after Bonferroni corrections. Results were similar in the measurements preformed on day 2 (data not shown).

Impact of methods for data analysis on inter-day reliability

Repeated-measures ANOVA indicated no significant effect of method for data analysis in absolute value of inter-day RMR and RER differences on both CCM and MGU metabolic carts (Figure 7, all P>0.2). Results remained unaltered when using inter-day percentages instead of absolute values (all P>0.2, data not shown).

Table 7. Resting metabolic rate (RMR) and respiratory exchange ratio (RER) inter-day reliability across methods for data analysis by metabolic cart (CCM and MGU).

	CMM (n=16)			MGU (n=17)		
	Bias	(Lower limit ; Higher limit)	P	Bias	(Lower limit ; Higher limit)	P
RMR (Kcal/day)						
1-5 min	51	(-385 ; 488)	0.364	87	(-490 ; 665)	0.231
6-10 min	48	(-396 ; 493)	0.396	45	(-539 ; 629)	0.533
11-15 min	53	(-398 ; 505)	0.361	69	(-502 ; 640)	0.334
16-20 min	58	(-373 ; 488)	0.3	59	(-513 ; 631)	0.408
21-25 min	61	(-361 ; 483)	0.267	77	(-508 ; 662)	0.295
26-30 min	46	(-424 ; 517)	0.443	67	(-507 ; 641)	0.352
1-30 min	53	(-376 ; 482)	0.339	67	(-499 ; 634)	0.342
6-30 min	53	(-380 ; 486)	0.34	63	(-508 ; 635)	0.374
6-25 min	55	(-371 ; 481)	0.318	62	(-512 ; 637)	0.383
3 min SSt*	50	(-375 ; 475)	0.359	77	(-489 ; 644)	0.277
4 min SSt*	48	(-388 ; 484)	0.39	83	(-485 ; 651)	0.244
5 min SSt*	53	(-382 ; 487)	0.347	75	(-468 ; 619)	0.27
10 min SSt*	55	(-358 ; 468)	0.301	69	(-534 ; 672)	0.361
RER						
1-5 min	0.01	(-0.09 ; 0.10)	0.549	0	(-0.11 ; 0.12)	0.786
6-10 min	0	(-0.07 ; 0.07)	0.934	0.01	(-0.08 ; 0.09)	0.578
11-15 min	0	(-0.07 ; 0.08)	0.821	0.01	(-0.08 ; 0.10)	0.362
16-20 min	0	(-0.07 ; 0.07)	0.989	0	(-0.10 ; 0.10)	0.88
21-25 min	0.01	(-0.06 ; 0.09)	0.226	0	(-0.10 ; 0.10)	0.946
26-30 min	0.02	(-0.21 ; 0.25)	0.478	0	(-0.09 ; 0.09)	0.828
1-30 min	0.01	(-0.06 ; 0.07)	0.417	0	(-0.08 ; 0.08)	0.746
6-30 min	0.01	(-0.07 ; 0.08)	0.469	0	(-0.08 ; 0.09)	0.761
6-25 min	0	(-0.05 ; 0.06)	0.627	0	(-0.08 ; 0.09)	0.748
3 min SSt*	0.01	(-0.11 ; 0.12)	0.658	0.01	(-0.09 ; 0.10)	0.641
4 min SSt*	0.02	(-0.07 ; 0.1)	0.134	0	(-0.10 ; 0.10)	0.945
5 min SSt*	0.01	(-0.07 ; 0.1)	0.206	0	(-0.10 ; 0.09)	0.693
10 min SSt*	0.01	(-0.05 ; 0.06)	0.451	0.01	(-0.07 ; 0.09)	0.459

Data are mean bias (Day2 - Day1) and the 95% limits of agreement (mean difference ±1.96 standard deviation of the difference). P from paired T-test for Day1 vs. Day2. *Steady state time (SSt) period is defined as the period (3, 4, 5, or 10 minutes) with the lowest coefficient of variance for VO2, VCO2, RER, and VE (i.e. the most stable period of n minutes).

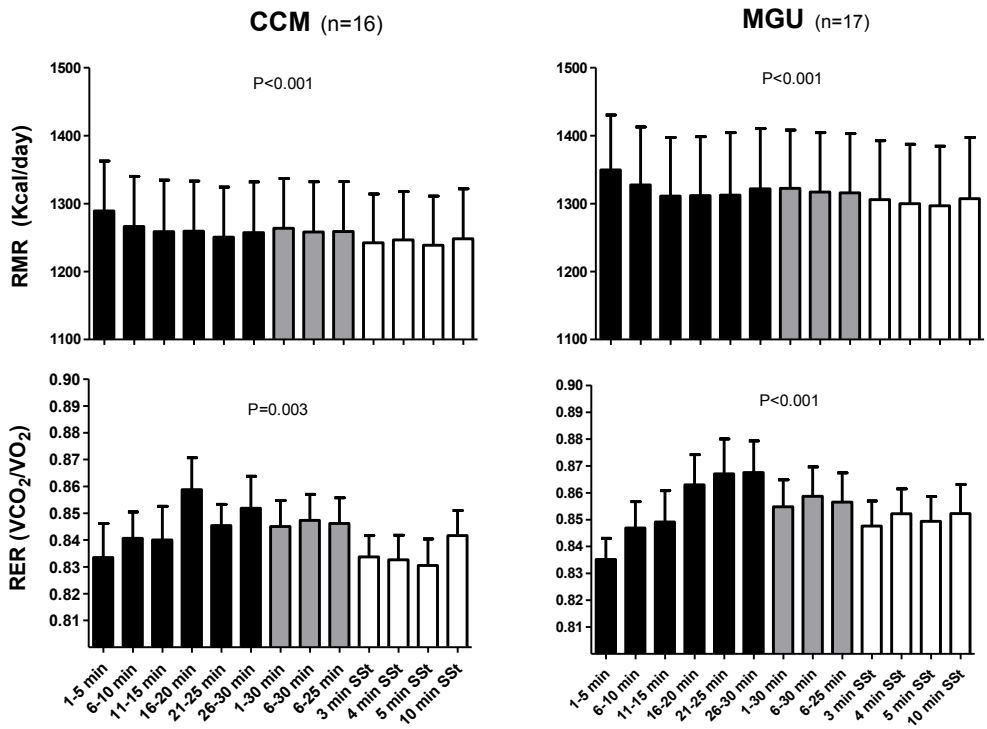

Figure 6. Day 1 resting metabolic rate (RMR) and respiratory exchange ratio (RER) measurements across methods for data analysis (steady state time and time interval) in the CCM and MGU metabolic carts. Black columns represent time intervals of 5 minutes; Grey columns represent time intervals for longer time periods; and White columns represent steady state time (SSt) periods. SSt is defined as the period (3, 4, 5, or 10 minutes) with the lowest coefficient of variance for VO_2, VCO_2, RER, and VE (i.e. the most stable period of *n* minutes). P from analysis of variance.

Table 7 shows inter-day mean bias (Day2 - Day1) and the 95% limit of agreement (mean difference±1.96 standard deviation of the difference) for every method for data analysis with the CCM and MGU metabolic carts. Paired *t*-test showed no significant RMR or RER inter-day mean differences in any of the methods for data analysis (all P>0.1). The limits of agreement were quite similar across methods, and no method presented the narrowest limits of agreement for all analysis. We observed that *10 min SSt*, *5 min SSt*, *6-25 min*, and *10 min SSt* presented the narrowest limits of agreement for RMR-CCM, RMR-MGU, RER -CCM, and RER -MGU, respectively.

Inter-day reliability across methods for data analysis in participants achieving SS vs. participants not achieving SS

Table 8 shows the comparisons between participants who achieved SS and those who did not on inter-day differences in RMR and RER in each method for data analysis and for both CCM and MGU metabolic carts. All participants, except one, achieved the SS criteria when following the *3, 4*, and *5 min SSt* method for data analysis. There were no significant mean differences between participants who achieved the SS criteria and those who did not, except in RMR-MGU following the *6-10 min* method (98±108 vs. 296±180 Kcal/day,

respectively, P=0.027). Results were similar when the SS criteria were based on just VO_2 and VCO_2 CV (data not shown).

Table 8. Resting metabolic rate (RMR) and respiratory exchange ratio (RER) inter-day reliability across methods for data analysis between participants who achieved steady state (Steady State) and participants who did not (non-Steady state), and by metabolic cart (CCM and MGU).

	CMM (n=16)							MGU (n=17)						
	n	*Steady state		n	non-Steady state		P	n	*Steady state		n	non-Steady state		P
RMR (Kca/day)														
1-5 min	3	270	(258)	13	138	(114)	0.473	3	147	(153)	14	245	(198)	0.44
6-10 min	2	374	(347)	14	121	(115)	0.488	6	98	(108)	11	296	(180)	**0.027**
11-15 min	5	80	(54)	11	191	(190)	0.226	10	186	(191)	7	273	(178)	0.361
16-20 min	4	203	(297)	12	135	(95)	0.68	7	214	(210)	10	230	(168)	0.863
21-25 min	5	208	(244)	11	136	(84)	0.552	6	247	(266)	11	210	(159)	0.824
26-30 min	8	219	(213)	8	131	(61)	0.293	6	196	(263)	11	215	(176)	0.858
1-30 min	5	207	(241)	11	128	(105)	0.366	8	170	(215)	9	264	(151)	0.306
6-30 min	6	190	(226)	10	131	(108)	0.483	9	196	(224)	8	245	(144)	0.607
6-25 min	6	182	(229)	10	126	(110)	0.514	9	203	(221)	8	247	(138)	0.633
3 min SSt*	16	140	(164)	0			NC	16	226	(193)	1	80		NC
4 min SSt*	16	150	(161)	0			NC	16	233	(187)	1	144		NC
5 min SSt*	15	163	(158)	1	83		NC	16	213	(190)	1	120		NC
10 min SSt*	11	157	(179)	5	115	(91)	0.634	14	254	(206)	3	159	(29)	0.129
RER														
1-5 min	3	0	(0)	13	0.04	(0.03)	0.077	3	0.02	(0.02)	14	0.04	(0.04)	0.458
6-10 min	2	0.02	(0.02)	14	0.03	(0.02)	0.396	6	0.04	(0.02)	11	0.04	(0.03)	0.958
11-15 min	5	0.02	(0.02)	11	0.03	(0.02)	0.334	10	0.03	(0.03)	7	0.04	(0.02)	0.441
16-20 min	4	0.02	(0.02)	12	0.03	(0.02)	0.283	7	0.03	(0.03)	10	0.04	(0.04)	0.284
21-25 min	5	0.03	(0.02)	11	0.04	(0.02)	0.821	6	0.03	(0.02)	11	0.04	(0.03)	0.068
26-30 min	8	0.05	(0.02)	8	0.07	(0.14)	0.61	6	0.03	(0.02)	11	0.04	(0.03)	0.544
1-30 min	5	0.01	(0.02)	11	0.03	(0.02)	0.197	8	0.03	(0.02)	9	0.04	(0.02)	0.348
6-30 min	6	0.02	(0.02)	10	0.03	(0.03)	0.414	9	0.03	(0.02)	8	0.04	(0.03)	0.39
6-25 min	6	0.02	(0.02)	10	0.02	(0.01)	0.734	9	0.03	(0.02)	8	0.04	(0.03)	0.417
3 min SSt*	16	0.04	(0.04)	0			NC	16	0.03	(0.03)	1	0.03		NC
4 min SSt*	16	0.03	(0.03)	0			NC	16	0.04	(0.03)	1	0.07		NC
5 min SSt*	15	0.03	(0.03)	1	0.07		NC	16	0.03	(0.03)	1	0.02		NC
10 min SSt*	11	0.03	(0.01)	5	0.02	(0.02)	0.522	14	0.04	(0.03)	3	0.05	(0.02)	0.799

Data are presented as absolute mean differences between day 1 and day 2 (e.g. |RMR Day1 – RMR Day2|) and (standard deviation). P from paired T-Test comparing participants who achieved steady state (SS) and participants who did not. "NC": Not computable. *Steady state (SS) is defined as the time period where VO2, VCO2, VE vary by <10% and RER vary by <5%. When these criteria are not met, measurement is defined as non-SS. **Steady state time (SSt) period is defined as the period (3, 4, 5, or 10 minutes) with the lowest coefficient of variance for VO2, VCO2, RER and VE (i.e. the most stable period of n minutes).

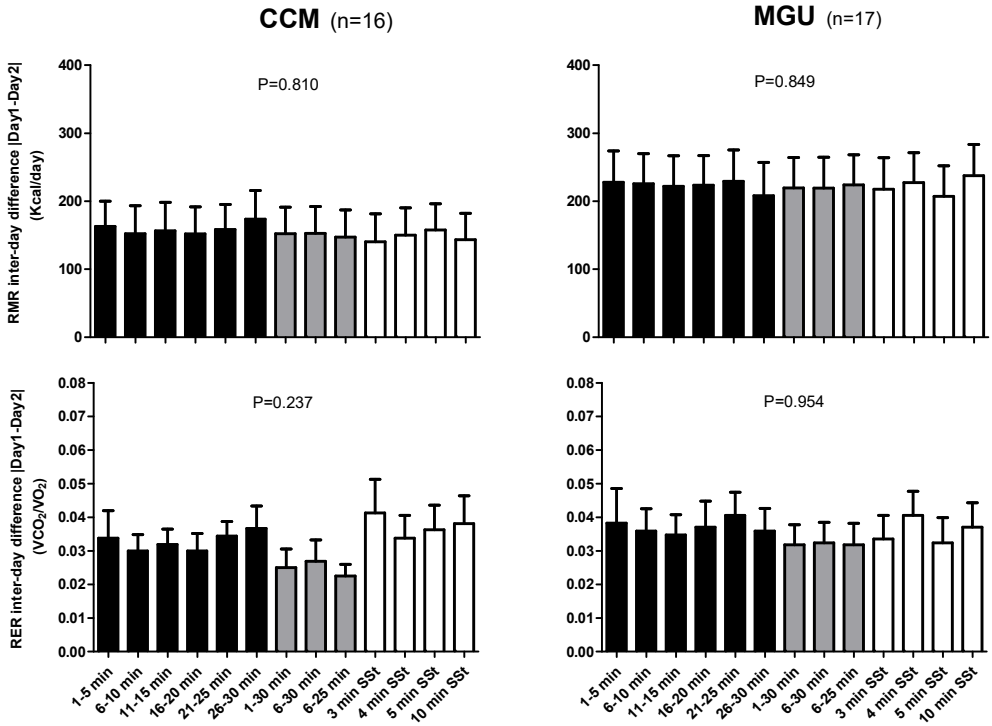

Figure 7. Inter-day reliability of resting metabolic rate (RMR) and respiratory exchange ratio (RER) across methods for data analysis (steady state and time interval) in the CCM and MGU metabolic carts. Y axis represents absolute values of the inter-day differences (e.g. |RMR Day1 – RMR Day2|). Black columns represent time intervals of 5 minutes; Grey columns represent time intervals for longer time periods; and White columns represent steady state time (SSt) periods. SSt is defined as the period (3, 4, 5, or 10 minutes) with the lowest coefficient of variance for VO2, VCO2, RER, and VE (i.e. the most stable period of n minutes). P from analysis of variance.

DISCUSSION

The main findings of this study suggest that: i) RMR and RER measurements are lower when following SSt methods than when following TI methods in young healthy adults using the CCM or MGU metabolic carts. Although no significant differences were found between different lengths of SSt, *5 min SSt* seems to present the lowest RMR and RER values; ii) there are no differences on the inter-day reliability across methods for data analysis (TI and SSt), and there is no systematic bias when comparing RMR and RER day 1 and day 2 measurements; iii) inter-day reliability seems to be comparable between participants who achieved the SS and participants who did not. Of note is that the results were consistent independently of the metabolic cart used. Taken together, these findings suggest that *5 min SSt* should be the method of choice, and that not achieving SS should not be an inclusion criterion in an IC study with young adults using either the CCM or MGU metabolic cart.

We observed that *5 min SSt* was the method which obtained the lowest RMR value in both metabolic carts, and the lowest RER in one of the metabolic carts (CCM). These results concur with those reported by Irving et al. [10]. They reported that RMR values

obtained by *5 min SSt* method was lower than values obtained with TI methods of several lengths. However, there are some differences between our study and the one by Irving et al. [10]: (i) they excluded participants who did not achieve the SS, (ii) they did not include other SSt periods in their analysis, and (iii) they did not compare RER values between methods for data analysis. Nevertheless, as RMR is considered to be the lowest EE on an awake person, our results also suggest that *5 min SSt* may provide the most valid RMR measurement. Nonetheless, it should be noted that when comparing mean RMR measurements between different SSt methods, maximum differences were of 20 Kcal/day, which may not be of clinical relevance, and no statistical differences were found. Reeves et al. [11] also showed similar RMR differences when comparing *3 min SSt*, *4 min SSt*, and *5 min SSt*, which also concur with our results.

Interestingly, Reeves et al. [11] showed that only 54% of the participants were able to achieve SS on *5 min SSt*, while Irving et al. [10] reported that 84% of participants achieved SS on *5 min SSt*. Horner et al. [16] observed that 93% of participants achieved SS on *5 min SSt* (considering a CV<10% for VO_2, RER, and VE) and that 47% of participants achieved the SS on *10 SSt* in 30 min of measuring. These results are in agreement with our study. We observed that 93.7% and 94.1% (CCM and MGU, respectively) of participants achieved the SS on *5 min SSt* on both testing days, and only 68.7% and 82.3% (CCM and MGU, respectively) achieved the SS on *10 min SSt* on both testing days. Surprisingly, we found a higher percentage of participants that achieved SS than most of previous studies, except for Horner et al. [16]. It is to note that Reeves et al. [11] included patients with cancer, and that the participants in Irving et al. [10] study were considerably older than those taking part in our study. Thus, it is plausible that both health status [9], sex [11], and age influence the ability to achieve SS. Further studies are needed to confirm this hypothesis.

A high RMR reliability is important in order to be able to detect changes resulting from an intervention or for between-individual comparisons on a cross-sectional study [12, 13]. Haugen et al. [21] reported 79±11 Kcal/day absolute inter-day RMR differences when measuring healthy individuals with a canopy system (model 2900 Metabolic Cart; SensorMedics). Cooper et al. [19] showed a 10.9% mean inter-day variance of RMR measured with an older MGU model. In our study, the inter-day variability following the *5 min SSt* method was 158±154 Kcal/day (13.5±15.3%) for the CCM, and 219±185 Kcal/day (18.3±17.2%) for the MGU (Figure 8). Of note is that the reliability was similar when using different methods for data analysis (TI and SSt). Future studies are needed to confirm if these results also apply to other metabolic carts such as those used by Haugen et al. [21] or Cooper et al. [19].

Although factors influencing RMR inter-day reliability have been explored in several studies [19,21], not all of them have also studied RER inter-day reliability [21]. RER equally depends on VCO_2 and VO_2, whereas RMR depends mainly on VO_2. Consequently, using different methods for data analysis could have a different impact on RMR and RER inter-day reliability. In a study comparing six metabolic carts, Cooper et al. [19] showed that RER inter-day reliability was considerably better than RMR inter-day reliability. Indeed, there were no differences between the RER inter-day reliability obtained with the gold-standard metabolic cart (Deltatrac metabolic monitor), whereas important differences between metabolic carts were found for RMR inter-day reliability [19]. Taken together, these findings [19] suggest than RER has a better inter-day reliability than RMR. In line with this,

we found that RER inter-day reliability was not influenced by the selected method for data analysis. Nonetheless, it should be noted that limits of agreement of inter-day RER differences (Table 7) might not be considered clinically acceptable. It is to note that we did not control the composition of previous meals, which could affect RER inter-day reliability. In addition, RER inter-day reliability has been shown to be slightly worse in IC performed with a MGU metabolic cart (an older model than the one used in our study) than with several other metabolic carts [19], which could also explain why we found these high inter-day RER differences.

Achieving SS is generally considered necessary to obtain a valid measure of RMR and RER [9–11,15]. However, we found that participants who achieved SS did not consistently present higher inter-day RMR or RER reliability than participants who did not achieve SS in any of the methods used for data analysis. Although an unequal distribution of participants in both groups (SS vs. non-SS) hampers deeper analysis, our results suggest that achieving SS does not improve inter-day reliability. These findings concur with those by Horner et al. [16]. They showed no higher repeatability on participants achieving SS than in those not achieving SS when analyzing data following the TI methods. If confirmed, these results should be considered in future IC studies, as it might be that excluding participants who do not achieve SS, and consequently loosing statistical power, has no advantage in terms of inter-day reliability.

The results of this study should be considered with caution as there are some limitations. Participants were healthy young adults, and we do not know if these findings can be extended to older or unhealthy people. We strictly controlled the fasting time (8 hours) prior to IC measurements, which is considered a mandatory condition to measure RER [22]. However, the composition of previous meals was not standardized, which affect the RER measurements [23]. Whereas our results are similar when using the CCM and MGU metabolic carts, we do not know if our findings apply to other metabolic carts, or even to other gases collection systems such as canopy which may affect the RMR estimation (e.g. canopy) [24,25]. Due to the relatively low sample size, we were not able to analyze the data in men and women separately. Further studies with larger sample sizes are needed to confirm the impact of achieving SS on inter-day RMR and RER reliability.

In summary, our findings suggest that inter-day RMR and RER reliability is not influenced by the use of different methods for data analysis (TI and SSt) and that it is not better in participants who achieved SS. This finding implies that participants who do not achieve SS should not be excluded from data analysis. Moreover, our data confirm the use of the *5 min SSt* as the optimal method for analyzing RMR and RER from IC. The *5 min SSt* presented the lowest RMR value, and the proportion of participants able to achieve SS following this method was higher than with other methods for data analysis. These findings are further reinforced by the fact that the results are similar when using two different metabolic carts.

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Estimation of non-shivering thermogenesis and cold-induced nutrient oxidation rates: Impact of method for data selection and analysis

STUDY 2

BACKGROUND

CIT can be broadly divided into shivering and non-shivering thermogenesis [1], although both processes can occur concomitantly [2]. CIT and the associated changes in nutrient oxidation rates are commonly measured by IC [3]. IC data are quite variable minute by minute, and methods for data selection based on SS periods are often necessary to minimize individuals' and instrument's variability [3–8]. Alternatively, selection of predefined (i.e. not considering data variability) TI is commonly made [4]. Besides how to select data, it is often necessary to compute CIT as a unique value to be used in cross-sectional studies, such as to study the association between BAT and CIT [9]. Therefore, investigators have used AUC calculations and/or the difference between EE at the end of cold exposure and baseline RMR (Last-RMR) [6,10,11]. These methods for data selection (i.e. SS or TI) and analysis (i.e. AUC or Last-RMR) significantly impact on RMR or MIT estimations [4–7]. However, the impact of the chosen method for data selection and data analysis on the overall measure of CIT and CI-NUTox rates is largely unknown.

The aim of the present study was to analyze the impact of methods for data selection (TI and SS) and methods for data analysis (AUC and Last-RMR) on measures of cold-induced EE and nutrient oxidation rates. Despite large scientific interest in CIT during the last decade [12–14], to our knowledge, there are no studies evaluating the impact of various methodologies on the measurement of cold-induced EE in healthy human.

METHODS OVERVIEW

General overview

A total of 63 participants (45 women) participated in the study. The participants were part of the ACTIBATE study. During the CIT, visually detected and auto-reported shivering was recorded in 17 participants (n=16 women) who were therefore excluded from further analysis. In addition, participants with RER values higher than 1.1 or lower than 0.7 in any measure point, or a RER higher than 1.0 in RMR assessment, were also excluded from the analysis (n=2) [3]. Finally, a total of 44 participants were included in the EE analysis (Table 9). Mean fasting time was 9±3.7 hours. Of this sample, a total of 18 (n=13 women) strictly met the fasting time criterion for assessing nutrient oxidation rates (i.e. a fasting time of 6-8 hours) and were included in the nutrient oxidation rate.

Table 10 shows the methodology overview. For a more detailed methods description see "Methods" section.

Methods for data selection and analysis

IC data were averaged every minute and downloaded from the Breeze Suite (8.1.0.54 SP7) software. For RMR, we selected the most stable 5-minute period (i.e. the one with the lowest average of CVs for VO_2, VCO_2, VE, and RER) [7].

For CIT, we applied different methods for data selection and analysis. Methods for data selection refer to the way of processing the data obtained from the continuous IC instrument. After excluding the first 5 minutes of every 30-minute record [4], we used three different methods for data selection (Figure 8): i) TI every 5 minutes (*5min-TI*): mean

values of every consecutive 5-minute period (i.e. from the 6th to the 10th, from the 11th to the 15th, etc.); ii) The most stable 5-minute period of every forth part of the cold exposure (i.e. after dividing the cold exposure into 4 parts equal in length) (*5min-SS-4P*); iii) The most stable 5-minute period of every half part of the cold exposure (i.e. after dividing the cold exposure into 2 parts equal in length) (*5min-SS-2P*).

Table 9. Descriptive characteristics of the participants included in the energy expenditure analysis.

	All (n=44)		Male (n=15)		Female (n=29)	
Age (years)	22.1	(2.1)	22.4	(2.2)	22.0	(2.2)
BMI (kg/m^2)	25.6	(5.2)	27.9	(6.0)	24.4	(4.4)
Lean mass (kg)	42.7	(10.4)	54.6	(6.8)	36.4	(5.2)
Fat mass (kg)	27.2	(10.6)	29.9	(13.5)	25.8	(8.8)
Fat mass (%)	37.0	(8.0)	32.7	(8.5)	39.2	(6.9)
VO_2 (ml/min)	223	(39)	252	(45)	208	(23)
VCO_2 (ml/min)	192	(33)	216	(37)	178	(20)
RMR (kcal/day)	1564	(277)	1769	(324)	1459	(178)
RER	0.862	(0.054)	0.863	(0.048)	0.861	(0.057)

Data are presented as means (standard deviation). BMI: Body mass index; VO2: resting oxygen consumption; VCO2: resting carbon dioxide production; RMR: Resting Metabolic Rate; RER: resting respiratory exchange ratio.

In order to express the CIT as a single value, we used two different methods for data analysis (Figure 8): i) The AUC following the trapezoidal rule; and ii) the Last-RMR. Both methods for data analysis were expressed as a percentage of the baseline RMR.

Table 10. Study 2 methodology.

GENERAL INFORMATION	
General aim	To compare different CIT data treatments
Design	Repeated measures
Cohort and participants	ACTIBATE (n=44)
OUTCOMES INCLUDED IN THE STUDY	
Outcome	Variables included
CEIC	CIT Cold-induced CHOox Cold-induced FATox

CEIC: Cold exposure indirect calorimetry; CIT: Cold-induced thermogenesis; CHOox: carbohydrates oxidation; FATox: Fat oxidation.

VO_2 and VCO_2 for each selected data point were used to estimate EE, CHOox and fat FATox. EE was estimated through Weir´s abbreviated equation, not considering urinary nitrogen concentration [15]. For carbohydrates and fat oxidation estimations, we used Frayn´s equation, not considering urinary nitrogen concentration [16].

Statistical analysis

Results are presented as means ± SD, unless otherwise stated. The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 21.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set at <0.05.

A repeated-measures ANOVA was used to test differences in EE and nutrient oxidation rates across the selected data points following the different methods for data

selection and analysis. To compare CIT, cold-induced CHOox, and FATox estimations obtained with different combinations of methods for data selection and analysis, we conducted a two-factor (method for data selection * method for data analysis) ANOVA. Bonferroni corrections (automatically performed by the SPSS) were used to perform post hoc comparisons.

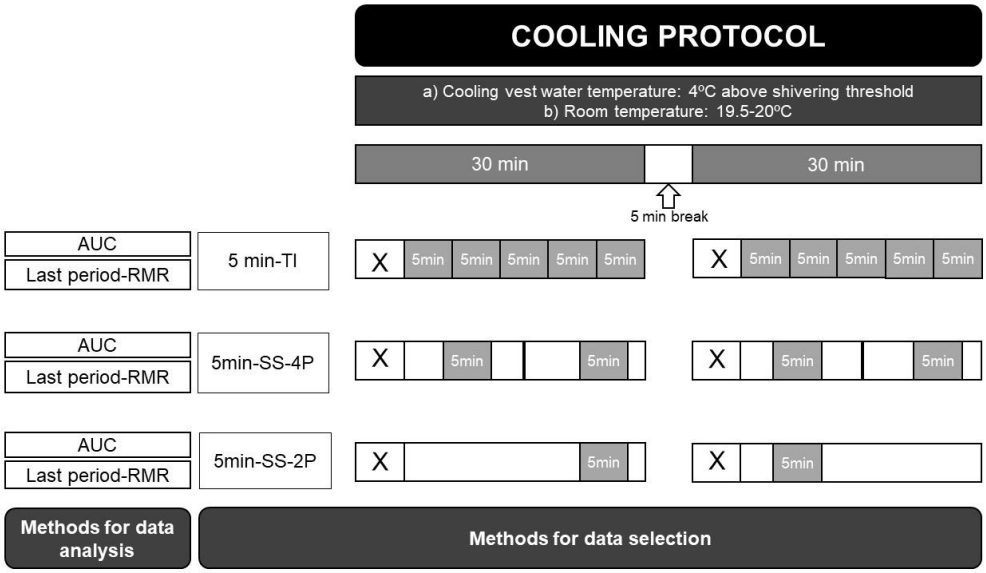

Figure 8. Cooling protocol, methods for data selection, and methods for data analysis. White rectangles in cooling protocol represent every 30 minutes of recorded gas exchange. Gray squares represent the 5-minute selected period within a specific recorded time (i.e. in the time interval method: average of every consecutive 5-minute period; in the steady state method: the 5 minute-period with the lowest average of coefficient of variances of: oxygen consumption, carbon dioxide production, respiratory exchange ratio, and minute ventilation). Crosses represent excluded gas exchange data (i.e. first 5 minutes of every 30-minute record). Vertical lines within white rectangles represent divisions of recorded data for the selection of the representative 5-minute period. TI: Time interval; SS: Steady State; min: minutes; 4P: Four periods; 2P: Two periods; RMR: Resting metabolic rate; AUC: Area under the curve.

RESULTS

Cold-induced thermogenesis

Figure 9 shows the EE dynamics during a mild cold exposure by method for data selection. EE was significantly increased by mild cold exposure, which was detected regardless of the method for data selection used (All $P<0.001$). Post-hoc comparisons showed that for all methods for data selection, EE was increased just after starting the cold-exposure (i.e. first data point analyzed) and remained unchanged until the end of the mild cold exposure.

Mean overall CIT estimation ranged from 11.6±10.0 to 20.1±17.2 %RMR depending on the methods for data selection and analysis used. Figure 10 shows the individual data for the over-all estimation of CIT by different combinations of methods for data selection and methods for data analyses.

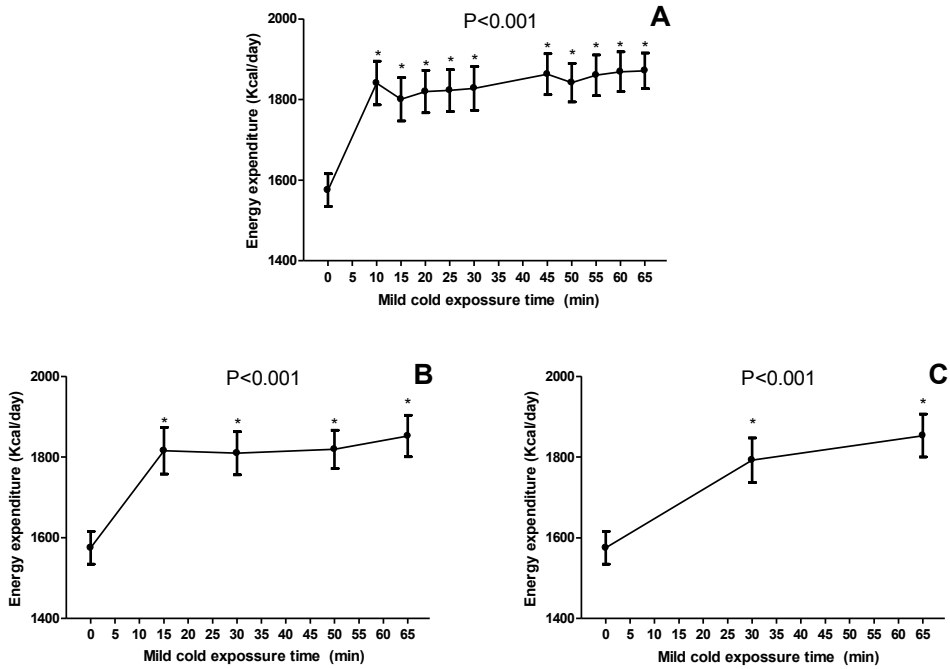

Figure 9. Energy expenditure (EE) during mild cold exposure by methods for data selection. Represented values are mean ± standard error. Panel A presents data obtained with the 5-minute time interval (TI) method; Panel B presents data obtained with the steady state (SS) method after dividing the cold exposure into 4 periods; Panel C presents data obtained with the steady state method after dividing the cold exposure into 2 periods. Min 0 represents the value obtained in the resting metabolic rate (RMR) period (baseline). *: Significantly different to baseline value. P value for repeated measures ANOVA. Min: minutes; Kcal: Kilocalories.

Figure 11 compares the mean overall CIT estimation obtained by the different methods for data selection and analysis. Both main effects (methods for data selection and methods for data analysis) were significant (all P<0.01) and no significant interaction effect (method for data selection * method for data analysis) was found (P=0.3). Mean overall CIT estimation was consistently higher with the Last-RMR than with the AUC in all methods for data selection (all paired comparisons P≤0.043). No differences in mean over-all CIT estimation were found between different methods for data selection when using the Last-RMR (all P=0.6). However, mean over-all CIT estimation varied across methods for data selection when using the AUC (P<0.001).

Cold-induced nutrient oxidation rates

Figure 12 shows CHOox and FATox dynamics during the mild cold exposure by method for data selection. There were significant changes in CHOox when selecting the data by *5min-TI* and *5min-SS-4P* (all P<0.015), but not with *5min-SS-2P* (P=0.547). Of note, differences between CHOox at 30 minutes and at the end of the mild cold exposure were only detected with *5min-TI*. FATox changes during the mild cold exposure were detected with all methods for data selection (All P<0.002). The highest FATox rate was observed at 30 minutes with *5min-TI* and *5min-SS-4P*, but not with *5min-SS-2P*. A reduction on FATox after minute 30 was only detected by *5min-TI*.

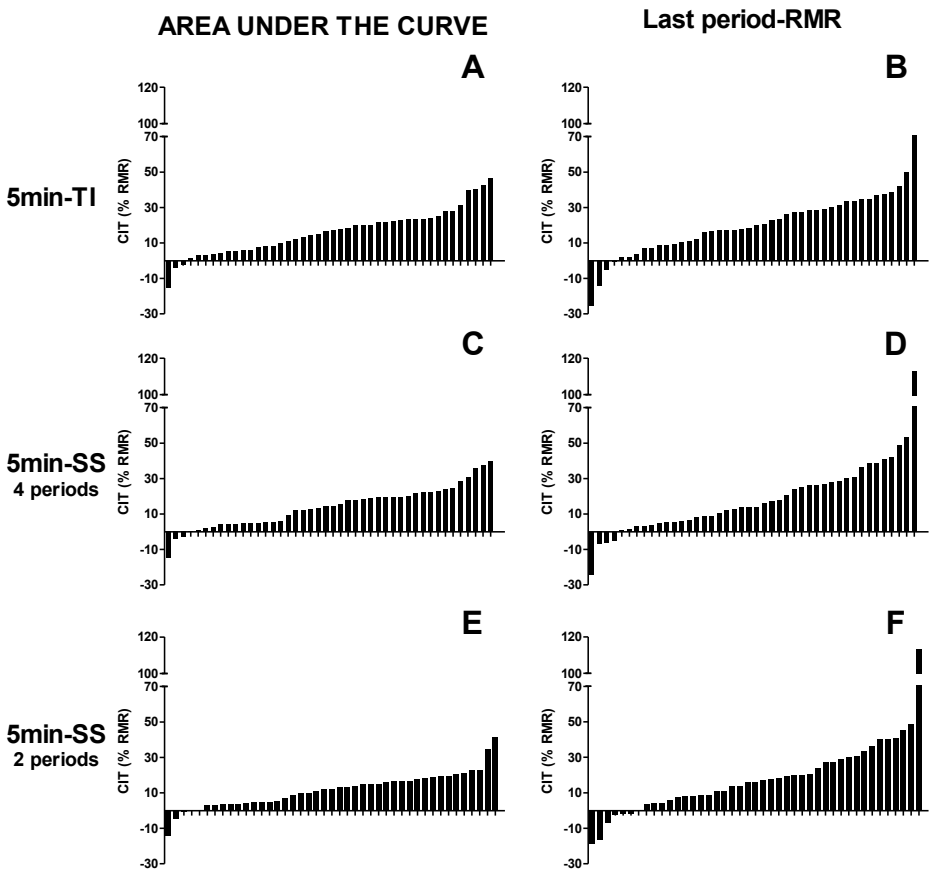

Figure 10. Individual data for over-all cold-induced thermogenesis (CIT) obtained with different methods for data selection and analysis. Panels A and B represent data obtained with the 5-minute time interval (TI) method for data selection. Panels C and D represent data obtained with the steady state (SS) method for data selection after dividing the cold exposure into 4 periods. Panels E and F represent data obtained with the SS method for data selection after dividing the cold exposure into 2 periods. Regarding the methods for data analysis, panels A, C, and E represent data obtained following the area under the curve method for data analysis, and panels B, D, and F represent data obtained from the difference between the last period of the cold exposure and the warm value. RMR: Resting metabolic rate.

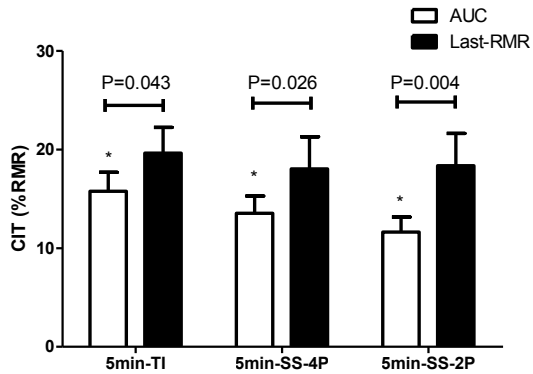

Figure 11. Comparisons between mean over-all cold-induced thermogenesis (CIT) obtained with different methods for data selection and analysis. Represented values are mean ± standard error. P values for paired t-test. *: significant differences with the rest of the AUC results after Bonferroni correction. RMR: Resting metabolic rate; AUC: Area under the curve; Last-RMR: Last period value minus RMR value.5min-TI: mean values of every consecutive 5-minute period; 5min-SS-4P: The most stable 5-minute period of every forth part of cold exposure; 5min-SS-2P: The most stable 5-minute period of every half part of cold exposure.

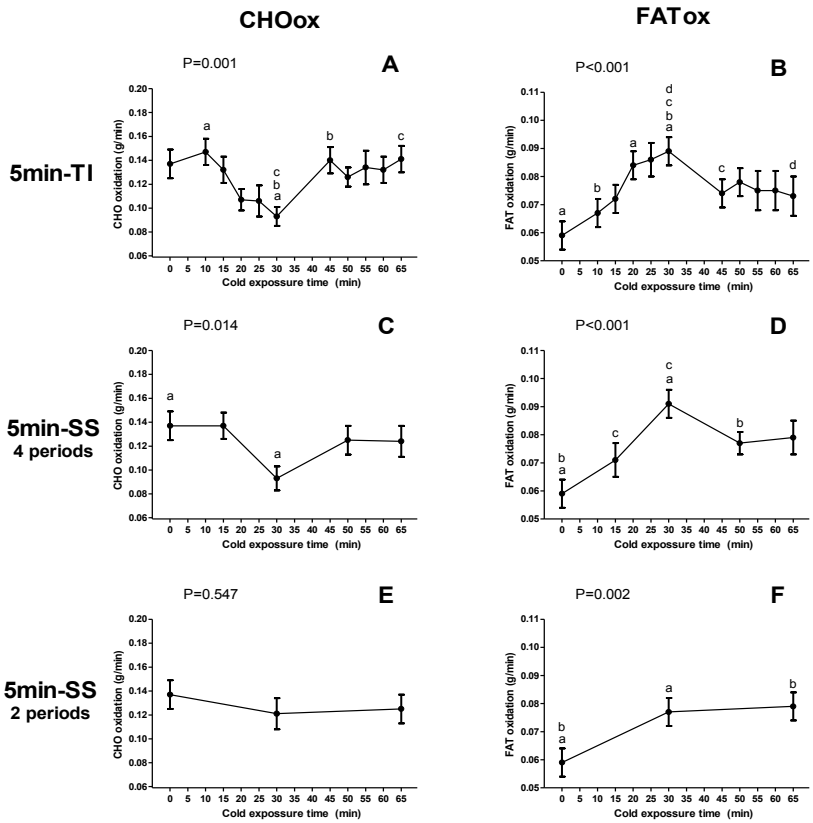

Figure 12. Nutrient oxidation rates during cold exposure by method for data selection. Panels A and B present data obtained with the 5-minute time interval (TI) method; panels C and D present data obtained with the steady state (SS) method after dividing the cold exposure into 4 periods; and panels E and F present data obtained with the steady state method after dividing the cold exposure into 2 periods. Represented values are mean ± standard error. P value for repeated measures ANOVA.

Equal lower-case letters indicate significant differences after Bonferroni correction. CHO: Carbohydrates. min: minutes; g: grams.

Regarding mean overall CI-NUTox estimation, no differences were found when comparing the methods for data selection (P=0.181), nor when comparing the methods for data analysis (P=0.328) (Figure 13).

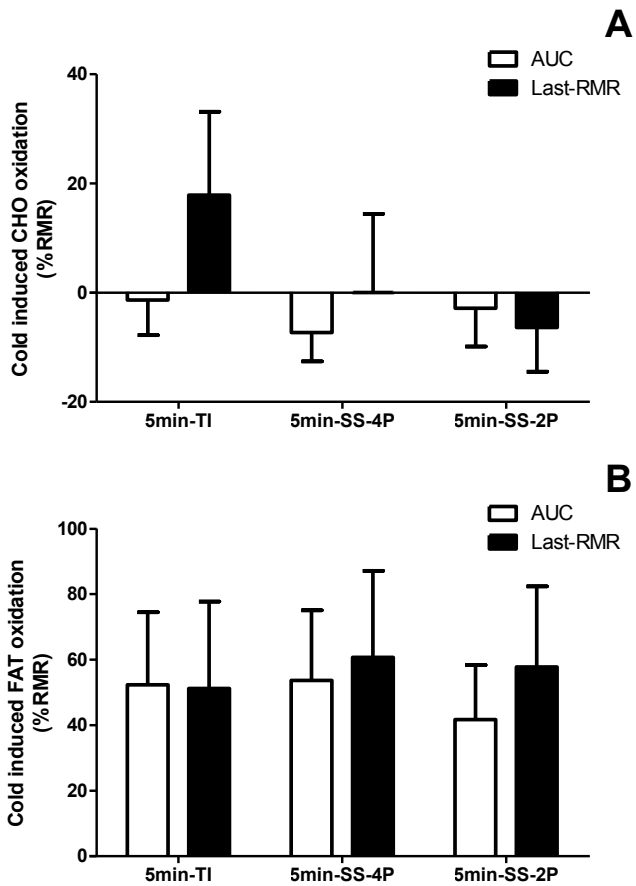

Figure 13. Comparisons between mean over-all cold-induced nutrient [carbohydrates (CHO) and fat (FAT)] oxidation rates obtained with different methods for data selection and analysis. TI: Time interval; SS: Steady State; RMR: Resting metabolic rate; AUC: Area under the curve; Last-RMR: Last period value minus RMR value. 5min-TI: mean values of every consecutive 5-minute period; 5min-SS-4P: The most stable 5-minute period of every forth part of the cold exposure; 5min-SS-2P: The most stable 5-minute period of every half part of the cold exposure.

DISCUSSION

This study analyzed the impact of methods for data selection (*5min-TI, 5min-SS-4P* and *5min-SS-2P*) in combination with two different methods for data analysis (AUC and Last-RMR) on estimations of cold-induced EE and nutrient oxidation rates during a 60-minute individualized mild cold exposure, designed to elicit maximum NST. The *5min-TI* and

5min-SS-4P methods for data selection seemed to be accurate enough to observe physiologically relevant phenomena, but not comparable for estimating over-all CIT and CI-NUTox. Regarding methods for data analysis, the AUC seemed to be less affected for data artefacts and be more representative in participants with a non-stable EE during cold exposure.

The selection of the method for data selection and analysis influences the estimations of RMR and MIT [3–8]. Therefore, it is expected that the selection of the method for data selection and analysis also influences the estimation of CIT and CI-NUTox. Regarding the methods for data selection, *5min-SS-2P* may not be an appropriate method, since, as a consequence of a lack of resolution, it does not allow to detect relevant physiological changes. In contrast, *5min-TI* allows to detect changes that no other method is able to detect (see Figure 12). However, for the RMR data selection, there is a consensus on the need of using a method based on the selection of a SS, as it is supposed not to be affected by artefacts, and to ensure a more valid measure [5,7,8]. Therefore, *5min-SS-4P* could be the method of choice. Our data supports that selection, especially when an over-all CIT estimation is made. In this case, the outcome obtained with the *5min-TI* method might be affected by artefacts (see Figure 13A), as previously argued [5,7,8]. Indeed, we observed a wider range of CIT values applying *5min-TI* (-14.9/46.2 %RMR) than *5min-SS-4P* (-14.8/39.9 %RMR) (see Figure 10). On the other hand, *5min-TI* might be the method of preference when studying the dynamics (i.e. changes during time of cold-exposure) of CIT or CI-NUTox, as it allows a more detailed insight (Figure 9 and 5). Standardizing the methods for data selection and analysis would allow between-studies comparability.

In relation to the methods for data analysis, the AUC resulted in a lower inter-individual variability than the Last-RMR (see figure 10). We observed a stable EE during the mild cold exposure, and consequently one could expect no differences between the AUC and the Last-RMR in over-all CIT estimation. In contrast, we observed large differences between the AUC and the Last-RMR over-all CIT estimation, with the Last-RMR reporting higher values (Figure 11). This could be explained by the fact that EE progressively increases during the mild cold exposure in some individuals while in others did not. This, together with the possibility of the Last-RMR methods to be influenced by artefacts (see outlier in Figure 10) would point to the AUC as the method of choice for over-all CIT estimation.

Observations on humans' CIT have reported huge inter-individual variability [12,17,18]. This is congruent with our results, where some individuals showed negative values of CIT (i.e. lower EE in cold than in RMR) while others even get more than 100% increase over RMR with some methods for data analysis. Many factors have been reported to contribute to inter-individual CIT difference. [12]. Here, we show that the method for data selection and analysis could have an important impact on inter-individual CIT variability estimations. This is in line with observations about the impact of the method for data selection and analysis on RMR estimations [3–5,7].

Our results should be considered with caution due to the presence of limitations. Firstly, we did not analyze urine nitrogen excretion, and therefore we could not correct the nutrient oxidation rates for protein oxidation. Although protein oxidation correction would have been desirable, it is not plausible to obtain different urine nitrogen

concentration in short intervals such as the periods that we have studied (i.e. ≤60 min). Secondly, although we selected a cooling protocol thought to ensure maximum NST, we cannot be sure of the relative contribution to CIT of shivering thermogenesis [2]. However, we excluded from the analysis participants who reported shivering or whose shivering was visually detected, and therefore it is probable than the contribution of NST is predominant in the included participants. Thirdly, we used two different metabolic carts which are not comparable and have relatively low reliability [19–21]. However, the within-subject design applied in this study reduce the impact of this limitation. Finally, our results only apply to young healthy individuals, and further studies are needed to confirm whether this also applies to older and unhealthy individuals.

In conclusion, the methods for data selection and analysis can have a profound impact on CIT and CI-NUTox estimations, and therefore, it is mandatory to unify it across scientific community to allow inter-study comparisons. Based on our findings, *5min-TI* should be considered the method of choice to study dynamics (i.e. changes across time) of CIT and CI-NUTox, while *5min-SS-4P* and AUC should be the method of choice when computing CIT and CI-NUTox as a single value.

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RESULTS AND DISCUSSION

SECTION 1

ROLE OF BROWN ADIPOSE TISSUE IN HUMAN ENERGY BALANCE

Energy expenditure and macronutrient oxidation in response to an individualized non-shivering cooling protocol

STUDY 3

BACKGROUND

CIT is mediated by both non-shivering and shivering physiological mechanisms [1]. Shivering thermogenesis is the EE necessary to cover involuntary skeletal muscle contractions in response to cold, while non-shivering thermogenesis refers to the energy consuming process not depending on muscle contraction such as uncoupling respiration in brown adipocytes mitochondria [2]. Although some have reported that shivering thermogenesis can increase EE up 5-fold above RMR, its uncomfortable nature, together with the loss of motor coordination, makes it an intolerable stimuli, and therefore, a non-plausible option to be used in clinical settings [2]. On the other hand, non-shivering thermogenesis produces moderate increases of EE, and estimations report a range between 0 and 30% of the RMR in young healthy adults[2]. Moreover, it has been argued whether non-shivering thermogenesis may be comfortable enough to be considered as a possible tool in the prevention and treatment of obesity [2]. Of note, shivering and non-shivering thermogenesis do not seem to occur in sequential phases but more as parallel phenomenon (i.e. even at light cold stimulation shivering thermogenesis seems to contribute to CIT) [3,4], and its relative contribution to CIT can vary within-individuals [5,6].

There is a large inter-individual variation in non-shivering thermogenesis and cold tolerance. Therefore, to study the responses to mild cold exposure, there is a need to individualize the cold stimulus to the individuals' cold tolerance [7–9]. Van der Lans et al. [7] proposed to first assess a shivering threshold (i.e. lowest external temperature without evoking externally observable and perceived shivering) as a reference point for adjusting the temperature at which an individual should be exposed. Although other methods have been proposed such as skin temperature clamping [9] or cold perception adjusting [10], the lowest tolerable temperature above the shivering threshold has been broadly accepted and used as a valid approach to induce BAT activation and CIT [8,11–14]. Some studies have however shown that skeletal muscle thermogenesis takes place with very mild cold stimuli before observing shivering [4]. As a result, the shivering threshold approach may not fully exclude the skeletal muscle shivering thermogenesis.

Research has traditionally spent more attention on the study of shivering thermogenesis [15,16], and important gaps remain regarding the human physiological responses to mild cold exposure in term of EE and metabolic fuels selection [1]. Thus, the present study aimed to describe the EE and macronutrient oxidation response to an individualized non-shivering cold exposure in young healthy adults.

METHODS OVERVIEW

General overview

Two different study cohorts took part in the present study. For the first cohort (ACTIBATE study), a total of 63 participants (45 women) participated in the study. In the ACTIBATE study, despite our careful assessment of a shivering threshold, visually detected and self-reported shivering was recorded in 17 (16 women) out of 63 participants, and those individuals were therefore excluded from further analysis. In addition, participants (2 men) presenting RER values higher than 1.1 or lower than 0.7 in any measure point, or a RER higher than 1.0 in RMR assessment, were also excluded from the analysis [17] (table 11).

For the second cohort (CIT-confirmatory), 13 participants (table 11) were recruited and evaluated between December 2017 and January 2018.

Table 11. Descriptive characteristics of the participants included in the energy expenditure analysis.

	CIT analyses (Study 1) (n=44)		NUTox analyses (Study 1) (n=18)		Confirmatory study (Study 2) (n=13)	
Sex (n women. %)	29	(65.9)	13.0	(72.2)	6.0	(46.2)
Age (years)	22.2	(2.2)	21.9	(2.0)	25.6	(3.0)
BMI (kg/m^2)	25.6	(5.3)	24.3	(4.6)	23.6	(2.4)
Lean mass (Kg)	42.7	(10.4)	40.4	(8.0)	45.7	(13.3)
Fat mass (Kg)	27.2	(10.6)	25.0	(9.6)	18.4	(3.8)
Fat mass percentage (%)	37.0	(8.0)	36.1	(7.0)	28.4	(6.6)
VO_2 (ml/min)	223	(39)	222	(33)	216	(41)
VCO_2 (ml/min)	192	(33)	186	(27)	179	(31)
RMR (Kcal/day)	1565	(278)	1554	(227)	1484	(286)
RER	0.862	(0.054)	0.842	(0.048)	0.833	(0.036)

Data are presented as means (standard deviation). BMI: Body mass index; VO2: resting oxygen consumption; VCO2: resting carbon dioxide production; RMR: Resting Metabolic Rate; RER: resting respiratory exchange ratio.

Table 12 shows the methodology overview. For a more detailed methods description see “Methods” section.

Table 12. Study 3 methodology.

GENERAL INFORMATION	
General aim	To study the effect of cold exposure on CIT
Design	Repeated measures
Cohort and participants	ACTIBATE (n=44), and CIT-confirmatory (n=13)
OUTCOMES INCLUDED IN THE STUDY	
Outcome	Variables included
CEIC	CIT Cold-induced CHOox Cold-induced FATox

CEIC: Cold exposure indirect calorimetry; CIT: Cold-induced thermogenesis; CHOox: carbohydrates oxidation; FATox: Fat oxidation.

CIT-confirmatory study methodology

In the CIT-confirmatory study, the participants came two days to the research center by bus or by car (i.e. with the minimum possible PA). Before both visits, participants were advised to (i) sleep as usual, (ii) refrain from any moderate (in the previous 24 hours) or vigorous (in the previous 48 hours) PA.

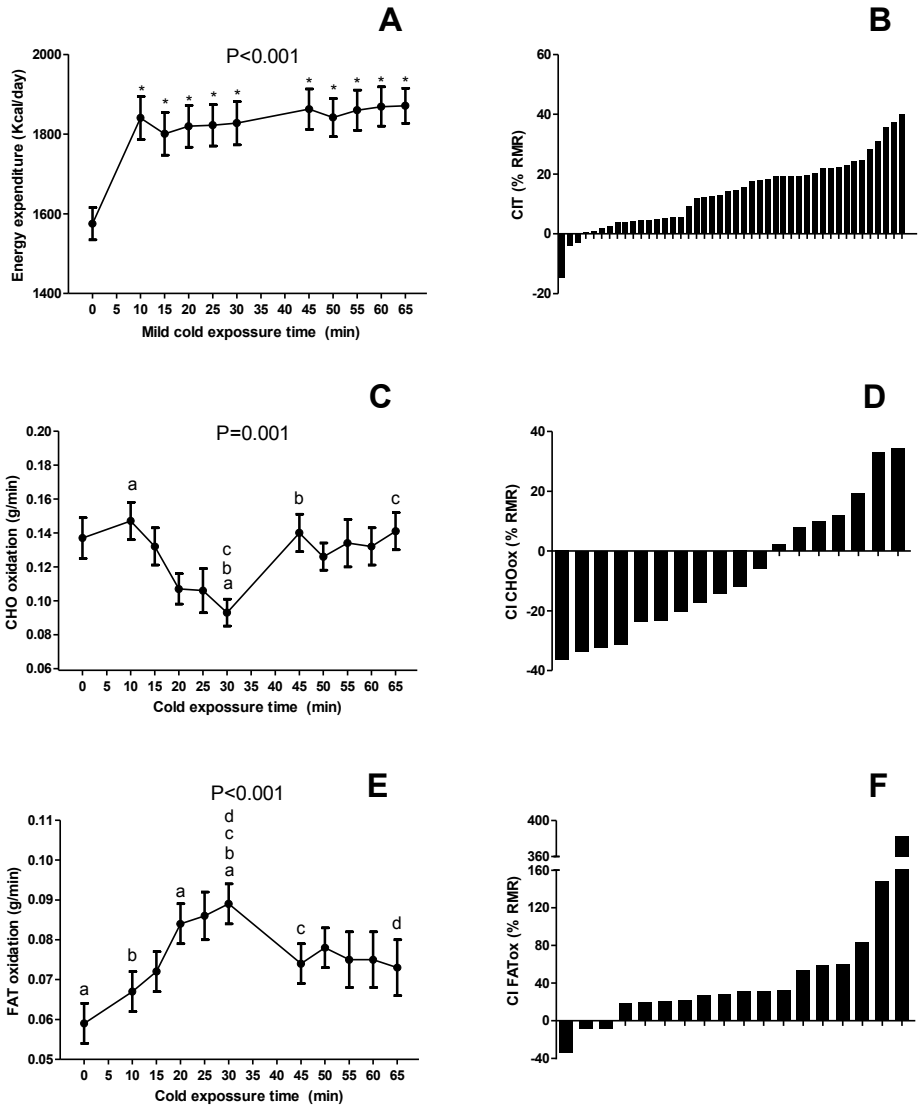

Figure 14. Energy expenditure and macronutrient oxidation rates during mild cold exposure (study 1). Represented values in panels A, C, and E, are mean ± standard error. Min 0 represents the value obtained in the resting metabolic rate (RMR) period (baseline). *: Significantly different to baseline value. Equal lower-case letters indicate significant differences after Bonferroni correction. P value for repeated measures ANOVA. Panels B, C, and D, present individual data for over-all cold-induced thermogenesis (CIT) and cold-induced (CI) macronutrient oxidation rates computed with the trapezoidal rule for area under the curve calculation. Kcal: Kilocalories; g: grams; min: minutes; CHO: Carbohydrates.

We used the same methodology described for the ACTIBATE study (see *The activating brown adipose tissue through exercise (ACTIBATE) study: Design and methodology* section), except that the participants were instructed to consume a meal equivalent to their estimated 30% TEE consisting on boiled rice, tomato sauce, and omelet, 10 hours before the CIT assessment. Moreover, they collected all produced urine from the standardized meal to the assessment start and immediately after the CIT

assessment to calculate the protein oxidation rate during baseline assessment and during the cold exposure, respectively. Moreover, in the CIT-confirmatory study, we measured total urine volume and urea concentration (Spinreact, Catalogue No. 283-17) in both urine samples. Thereafter, we estimated nitrogen urine levels from urea concentration following the equation "N(g/l) = 0.0065* Urea(mg/dl) + 1.2598" obtained from a linear regression including urea and nitrogen measured concentrations from 20 participants in a previous study (supplementary material).

Statistical analysis

Results are presented as means ± SD, unless otherwise stated. The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 21.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set at <0.05. A repeated-measures analysis of variance (ANOVA) was used to test differences in EE and macronutrient oxidation rates across the selected data points. Bonferroni corrections were used to perform post hoc comparisons.

RESULTS

Mild cold exposure significantly increased EE (P<0.001; Figure 14A), and post-hoc comparisons showed that EE was increased just after starting the cold-exposure (i.e. first data point analyzed) and remained unchanged until the end of the mild cold exposure. CHOox decreased after 30 minutes of mild cold exposure (P=0.001), although its level returned to the baseline after 40 minutes of mild cold exposure (Figure 14C). The highest FATox rate was observed at 30 minutes of cold exposure (P<0.001; Figure 14E). FATox progressively increased until minute 30, and later on, there was a decrease.

Mean overall CIT estimation was 13.9±11.6 %RMR (Range: -14.8/39.9 %RMR, Figure 14B). When translated to cumulative EE (i.e. the total amount of energy above the RMR expended during the whole 60-minute cold exposure), it resulted in 9.56±7.9 Kcal. Mean overall CI CHOox was -7.3±22.5%RMR (Range: -36.4/34.31 %RMR) and mean overall CI FATox was 53.6±90.9 %RMR (Range: -33.9/382.1 %RMR).

Figure 15 shows EE and macronutrient oxidation rates during a mild cold exposure in the Confirmatory-CIT study. Cold exposure increasde EE as in the ACTIBATE study (P>0.001; Figure 15A). The effect of cold exposure on both CHOox (Figure 15B) and FATox (Figure 15C) was also similar to ACTIABTE study.

DISCUSSION

We show that a 60-minute individualized mild cold exposure produced a moderate EE (9.56±7.9 Kcal) in young healthy adults. Interestingly, we observed a metabolic shift in time for sustaining CIT. CHOox decreased during the first 30 minutes of the cold exposure and recovered up to the baseline values to the end of the cold exposure. In contrast, FATox continuously increased during the first 30 minutes, and decreased in the second part of the cold exposure. Of note is that these results were replicated in two independent studies which further reinforce the findings.

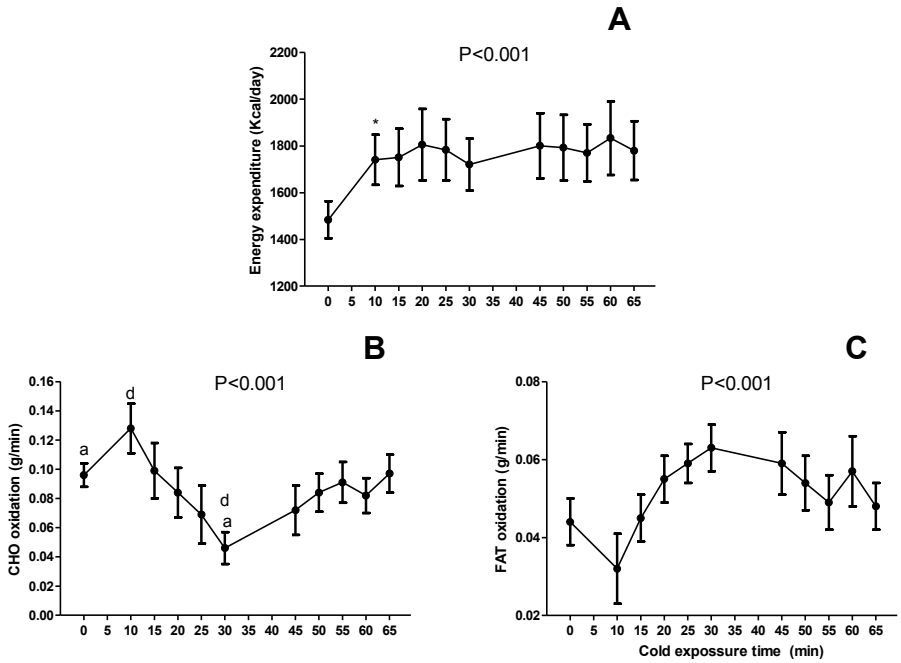

Figure 15. Energy expenditure and macronutrient oxidation rates during mild cold exposure in the confirmatory study (study 2). Represented values are mean ± standard error. Min 0 represents the value obtained in the resting metabolic rate (RMR) period (baseline). *: Significantly different to baseline value. Equal lower-case letters indicate significant differences after Bonferroni correction. P value for repeated measures ANOVA.

Mild cold exposure induces moderate increases in energy expenditure

Studies on humans' CIT have reported huge inter-individual variability [18–20], consistent with our own results with some individuals showing negative values of CIT (i.e. lower EE in cold than in RMR) while others even get up to 39% increase over RMR. Whether non-shivering cold exposure induce an stable EE from the beginning of the exposure is unclear [21]. We observed that when applying a personalized cooling protocol at a temperature near to the shivering threshold, maximum non-shivering thermogenesis seems to be elicited from the beginning of the cold exposure. This constant thermogenic rate concurs with other studies analyzing the EE elicited by shivering [22,23]. On the other hand, it is not clear whether the magnitude of NST is able to induce a significant negative energy balance [3,4,11]. We observed that even when applying a protocol designed to elicit maximum NST, all participants presented an EE below 1.4 MET (i.e. 40% of RMR increase). Taking into account that even a very low intensity exercise, such as walking, can elicit 2-3 METs [24], together with the fact that cold exposure seems to induce an hyperphagic response [25], it seems that cold exposures at temperatures eliciting low shivering and maximum NST are not a plausible stimuli to induce negative energy balance. It should also be noted that this mild cold stimulus also produces a considerable burden and discomfort for participants (despite only 9.56±7.9 Kcal were burned (above the RMR) after an hour at this uncomfortable situation). Nonetheless, if maximum NST were induced by pharmacological or nutraceutical agents [26,27], instead of cold exposure, and were

continuously elicited during 24h, our data would translate into 211.79±175.01 Kcal/day. Alternative mechanisms beyond energy balance [28,29] have been suggested to be mediating health promoting effects of chronic cold exposure [30–33], and therefore mild cold exposure might not be discarded as a possible therapeutic target (e.g. insulin sensitizing effects).

Mild cold exposure induced a shift on fuel oxidation

We observed a marked shift in macronutrient oxidation to sustain the thermogenic rate. Previous studies have reported similar metabolic shifts at a constant thermogenic rate in response to cold (in shivering conditions) [1,22,23], however, we observed a decrease in CHOox, which has not been reported before. Preserving muscle glycogen is considered to have a profound impact on cold endurance, and therefore on cold survival [1]. Consequently, fatty acids are the most sustainable fuel for thermogenic purposes. FATox is the predominant substrate for both non-shivering and shivering thermogenesis [1]. Therefore, it is biologically plausible that a shift to FATox is produced when thermogenic needs are not maximized, such as at the beginning of the cold exposure.

BAT is not the only responsible for NST in humans, yet skeletal muscle possibly contributes to NST even to a larger extent [4,11]. Although both BAT and skeletal muscle preferentially use fatty acids as energy fuel in non-shivering situations, BAT is probably more FATox-preferential, as more than 90% of its energy consumption relies on FATox [34,35], while skeletal muscle presents a more balanced nutrient uptake [1]. Therefore, it is plausible to speculate with the possibility of BAT the main contributor to CIT at initial stages of mild cold exposure while muscle contribution to CIT would increase progressively, therefore balancing the contribution of CHOox and FATox. This change on tissue role on sustaining CIT could be a consequence of BAT fatigue or from the increased demand for thermogenesis [1]. Of note, most of the studies analyzing the contribution of both skeletal muscle and BAT to CIT are based on static imaging techniques after a cold exposure period [11,15,32,33], and therefore, new studies examining the contribution of both tissues from the very beginning of the cold exposure are needed to confirm this hypothesis. If confirmed, it could have a profound impact on clinical practice, since it would mean that a very short mild cold exposure (i.e. 30 minutes) would be enough to activate and recruit BAT, which is thought to profoundly impact glucose and lipid homeostasis [21,28,33,36].

In agreement with previous studies [1], we observed a considerable high inter-individual variability in CI-NUTox. In human studies, different patterns of shivering (i.e. muscle recruitment) explain most of the inter-individual variability in CI-NUTox [1,22,23]. Since the protocol that we applied is considered to result in low shivering contribution to CIT, different patterns of shivering may not explain such a large inter-individual variability. Alternatively, inter-individual differences on tissues (BAT, skeletal muscle, and WAT) relative contribution to CIT (i.e. proportion of CIT being produce by each tissue) might partially explain such a high inter-individual difference [37].

There are some limitations that should be taken intro account when interpreting these findings. Firstly, we did not analyze urine nitrogen excretion in the ACTIBATE study, and therefore we could not correct the macronutrient oxidation rates for protein oxidation. In addition, in the ACTIBATE study, we did not strictly control fasting period and previous meal content. However, we performed a confirmatory study (i.e. Confirmatory-

CIT study) strictly controlling fasting time, previous meal content, and analyzing urine urea excretion and similar results were found. Secondly, although we selected a cooling protocol thought to ensure maximum NST, we cannot be sure of the relative contribution to CIT of shivering thermogenesis [4]. However, we excluded from the analysis participants who reported shivering or whose shivering was visually detected, and therefore it is probable than the contribution of NST is predominant in the included participants. Finally, our results only apply to young healthy individuals, and further studies are needed to confirm whether this also applies to older and unhealthy individuals.

In summary, a mild cold exposure at a temperature adjusted to elicit maximum NST induces a very modest increase in EE (<40% RMR; ≈1.4 METs), which is maintained constant during an hour. This suggest that mild cold exposure is not a feasible tool to induce negative energy balance in humans. Nonetheless, pharmacological or nutraceutical continuous induction of maximum NST might be a possible feasible target for weight management and needs to be further studied. Interestingly, we found a metabolic shift on macronutrient oxidation rates to sustain CIT by which FATox increased in parallel to a decrease in CHOox at the beginning of cold exposure. Later, an inverse tendency appeared by which CHOox increased and FATox slightly decreased. This may indicate that tissues playing a key role in CIT (i.e. BAT and muscles) could be different during the first stages of cold exposure than after 30 minutes of cold exposure.

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Association of brown adipose tissue with cold-induced thermogenesis and nutrient oxidation rates

STUDY 4

BACKGROUND

It is still not clear whether human BAT is able to produce a relevant increase in the EE in adult humans [1]. The main reason why BAT contribution to human EE is still unknown is the lack of technology to properly assess its contribution in vivo [2]. The most used technique to assess BAT volume and activity is the ^{18}F-FDG PET-CT scan [3]. Besides implicating high ionizing radiation exposure, the ^{18}F-FDG PET-CT scan's main limitation relates to the substrate preference of BAT. The ^{18}F-FDG radiotracer is a glucose analog. However, several studies have shown that brown adipocyte EE mainly relies on fatty acid oxidation [4,5]. Although other alternatives to the ^{18}F-FDG-PET-CT scan are being used, several limitations preclude the existence of a real gold-standard for in vivo BAT assessments in humans [2]. Among the alternatives to ^{18}F-FDG PET-CT, the skin temperature of the supraclavicular area has been used as an indirect marker of BAT activity, which would allow non-invasive and continuous assessments [6,7].

Besides the technical limitations to study the BAT contribution to human EE, it has been suggested that BAT could just be a minor contributor to CIT in humans, while skeletal muscle, by means of both shivering and non-shivering thermogenesis, could be the main effector of CIT [8–12]. Moreover, it has been suggested that not only skeletal muscle, but also white adipose tissue, could play a role in CIT [8,13]. To date, the are contradictory findings regarding the relative contribution of both human BAT and skeletal muscle to CIT [8–12,14–18], and more studies are needed to fully understand the relation of BAT and skeletal muscle activity with CIT. The relation of BAT and skeletal muscle activity with CI-NUTox has received much less attention and remains to be elucidated. Changes in the pattern of nutrient oxidation are related to overall metabolic health [19–21]. Thus, even if BAT or skeletal muscle non-shivering thermogenesis had a small impact on EE, they would still be very interesting therapeutic targets for human metabolic health improvements if they modified the substrate oxidation.

This study aimed to investigate the association BAT and skeletal muscle ^{18}F-FDG activity after a personalized cold exposure with CIT and CI-NUTox in young healthy adults. Additionally, we examined the association of supraclavicular skin temperature as a proxy of BAT activity with CIT and CI-NUTox rates.

METHODS OVERVIEW

General overview

We used data from two different cohorts. A total of 57 young healthy adults (23.0±2.4 years old; 25.1±4.6 kg/m^2; 35 women) participated in the present study (Table 13). Forty-four participants (29 women) were part of the ACTIBATE study. Only 18 out of these 44 participants met the required fasting time (6-8 hours) to be included in the analyses referred to CI-NUTox (Table 13). In addition, 13 participants were enrolled (Table 13) in the CIT-confirmatory study, which was conducted between December 2017 and January 2018.

Table 14 shows the methodology overview. For a more detailed methods description see "Methods" section.

Table 13. Descriptive characteristics of the participants included in the energy expenditure analyses.

	CIT analyses (ACTIBATE) (n=44)	NUTox analyses (ACTIBATE) (n=18)	No PET-CT (CIT-confirmatory) (n=13)
Sex (women, %)	29 (65.9)	13 (72.2)	6 (46.2)
Age (years)	22.2 (2.2)	21.9 (2.0)	25.6 (3.0)
BMI (kg/m^2)	25.6 (5.3)	24.3 (4.6)	23.6 (2.4)
Lean mass (kg)	42.7 (10.4)	40.4 (8.0)	45.7 (13.3)
Fat mass (kg)	27.2 (10.6)	25.0 (9.6)	18.4 (3.8)
Fat mass percentage (%)	37.0 (8.0)	36.1 (7.0)	28.4 (6.6)
RMR (kcal/day)	1565 (278)	1554 (227)	1484 (286)
BAT volume (ml)	94.4 (59.6)	74.29 (49.7)	
BAT SUV mean	4.29 (1.60)	4.24 (1.11)	
BAT SUV peak	14.13 (7.22)	13.40 (6.08)	
Muscle SUV peak	1.67 (0.33)	1.63 (0.33)	
Descending aorta SUV peak	0.92 (0.21)	0.83 (0.20)	

Data are presented as means (standard deviation). BMI: Body mass index; RMR: Resting metabolic rate; BAT: Brown adipose tissue; SUV: Standardized uptake value; CIT: Cold-induced thermogenesis; NUTox: Nutrient oxidation rates.

Table 14. Study 4 methodology.

GENERAL INFORMATION	
General aim	To study the association of BAT and skeletal muscle ^{18}F-FDG act. with CIT
Design	Cross-sectional
Cohort and participants	ACTIBATE (n=44), and CIT-confirmatory (n=13)
OUTCOMES INCLUDED IN THE STUDY	
Outcome	Variables included
PET-CT	BAT volume (-190/-10 HU; Ind. SUV threshold.) BAT SUVmean (-190/-10 HU; Ind. SUV threshold; SUV_{LBM}) BAT SUVpeak (-190/-10 HU; Ind. SUV $threshold_{LBM}$) BAT SUVmean (-190/-10 HU; Ind. SUV threshold; SUV_{BM}) BAT SUVpeak (-190/-10 HU; Ind. SUV $threshold_{BM}$) All muscles SUVpeak
CEIC	CIT Cold-induced CHOox Cold-induced FATox
SkTa	Mean skin temperature Subclavicular skin temperature Supraclavicular skin temperature

BAT: Brown adipose tissue; 18F-FDG: 18F-Fluorodeoxuglucose; CIT: Cold-induced thermogenesis; PET-CT: Positron emission tomography-Computerized tomography; Ind: Individualized; SUV: Standardized uptake value; BM: Body mass; LBM: Lean body mass; CEIC: Cold exposure indirect calorimetry; CHOox: carbohydrates oxidation; FATox: Fat oxidation; SkTa: Skin temperature assessment.

CIT-confirmatory study methodology

This study followed a similar procedure to ACTIBATE study, except for some minor differences. BAT and skeletal muscle ^{18}F-FDG activity was not assessed, so CIT-Confirmatory study only included two testing days. On the shivering threshold test (day 1)

the participants lay on a bed instead of being seated, and the fasting time before the CIT assessment was 10 hours. Additionally, the time between the shivering threshold test and the CIT assessment was 48 hours instead of 5-7 days.

Statistical analyses

The distribution of the variables was verified using the Shapiro–Wilk test, skewness and kurtosis values, visual check of histograms, Q-Q, and box plots. The descriptive statistics are presented as mean ± SD, unless otherwise stated. The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 21.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set at <0.05.

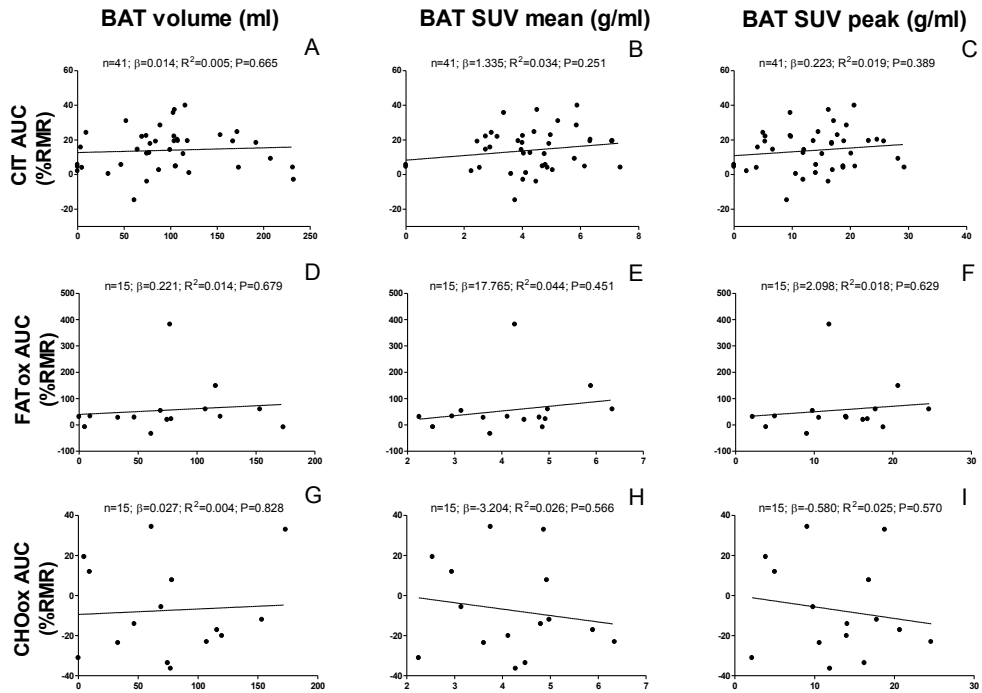

Figure 16. Associations of brown adipose tissue (BAT) 18F-FDG activity after a personalized cold exposure with cold induced thermogenesis (CIT) and cold-induced nutrient oxidation rates (Study 1). Unstandardized simple regression coefficient (β) and standardized coefficient of determination (R2). SUV: Standardized uptake value; AUC: Area under the curve; RMR: Resting metabolic rate; FATox: Fat oxidation rate; CHOox: Carbohydrates oxidation rate.

We used simple linear regression analyses to test the association of BAT and skeletal muscle ^{18}F-FDG activity after a personalized cold-exposure and supraclavicular temperature with CIT and CI-NUTox. We also used multiple linear regression models to test these associations adjusting by sex, BMI, the time of the day, and the date when CIT was assessed. Furthermore, we used repeated-measures ANOVA to study the cold-induced changes on skin temperature parameters. BAT and skeletal muscle ^{18}F-FDG activity after a personalized cold-exposure was only assessed in ACTIBATE study. Therefore, the CIT-confirmatory was only included in the analyses studying the association of the supraclavicular skin temperature with CIT and CI-NUTox.

RESULTS

The associations of BAT with CIT and CI-NUTox are shown in Figure 16. There was no association of BAT (volume: all P>0.68; mean activity: all P>0.25; maximal activity: all P>0.39) with CIT and NUTox. The results persisted after adjusting by sex, BMI, the time of the day, or the date when CIT was assessed. In addition, we analyzed whether using SUV_{LBM}, instead of SUV_{BM}, influenced the results [22], and no differences were found (data not shown).

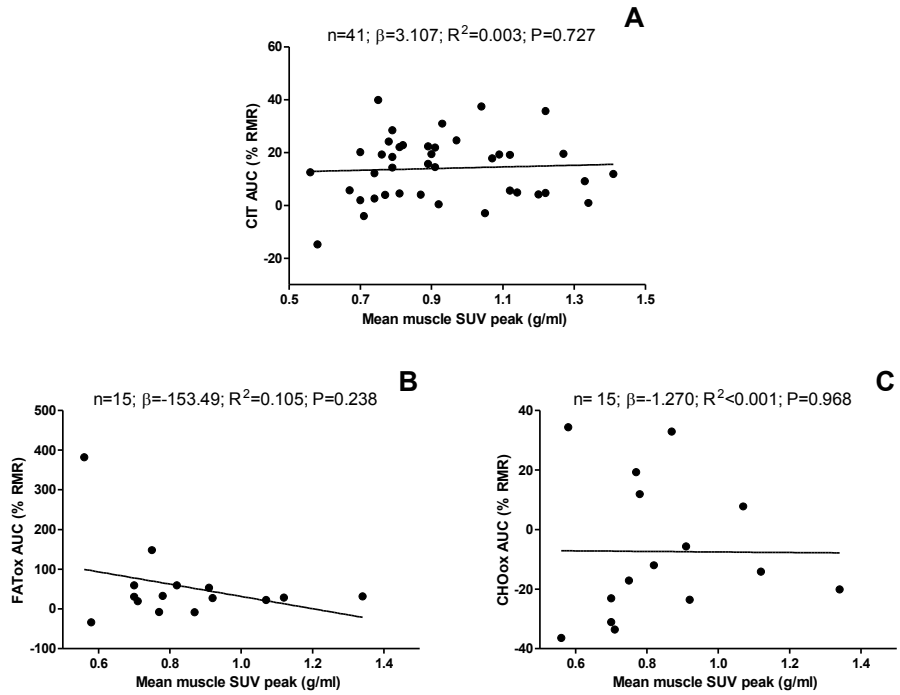

Figure 17. Association of skeletal muscle 18F-FDG activity after a personalized cold exposure with cold induced thermogenesis (CIT) and cold-induced nutrient oxidation rates (Study 1). Skeletal muscle 18F-FDG activity represents an average of the uptake in several skeletal muscles: paracervical muscles (cervical vertebrae 4), sternocleidomastoid, scalene, longus colli, trapezius, parathoracic muscles (Thoracic vertebrae 2), supraspinatus, subscapularis, deltoid, pectoralis major, and triceps brachii. Unstandardized simple regression coefficient (β) and standardized coefficient of determination (R2). SUV: Standardized uptake value; AUC: Area under the curve; RMR: Resting metabolic rate; FATox: Fat oxidation rate; CHOox: Carbohydrates oxidation rate.

Figure 17 shows the association of skeletal muscle ^{18}F-FDG activity after a personalized cold exposure with CIT and CI-NUTox. There were no associations either when using SUV_{BM} (all P>0.23) or SUV_{LBM} (data not shown). Adjusting the analyses by sex, BMI, the time of the day, and the date when CIT was assessed did not modify the results. Furthermore, we tested the association of CIT and CI-NUTox with the deep, cervical, and cold sensitive muscles activity, as they have been shown to respond differently to cold [8,23]. We found no significant association with any criteria for any group muscle (i.e. deep muscles, cervical muscles, and cold sensitive muscles). All these results remained when using skeletal muscle SUVmean instead of SUVpeak (data not shown).

Changes on mean, subclavicular, and supraclavicular skin temperature during CIT assessment are shown in Figure 18. The associations of cold-induced changes in supraclavicular skin temperature with CIT and CI-NUTox are shown in Figure 19 (including data of studies 1 and 2). We failed to observe any significant association (all P>0.09), which was unaffected when adjusting by sex, BMI, the time of the day, or the date when CIT was assessed. Similar results were found when using the skintemperature data at the end of the test instead of the cold-induced change (Δ). Moreover, neither the mean nor subclavicular skin temperature were associated with CIT or CI-NUTox.

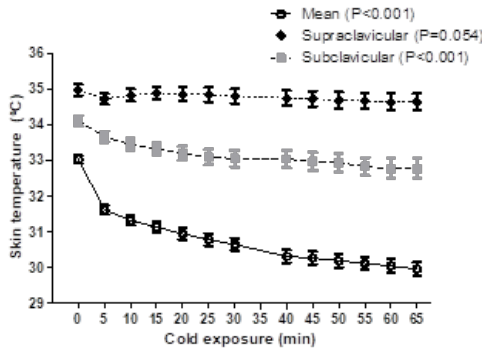

Figure 18. Skin temperature parameters during cold-induced thermogenesis. P for one-way analysis of variance. min: minute.

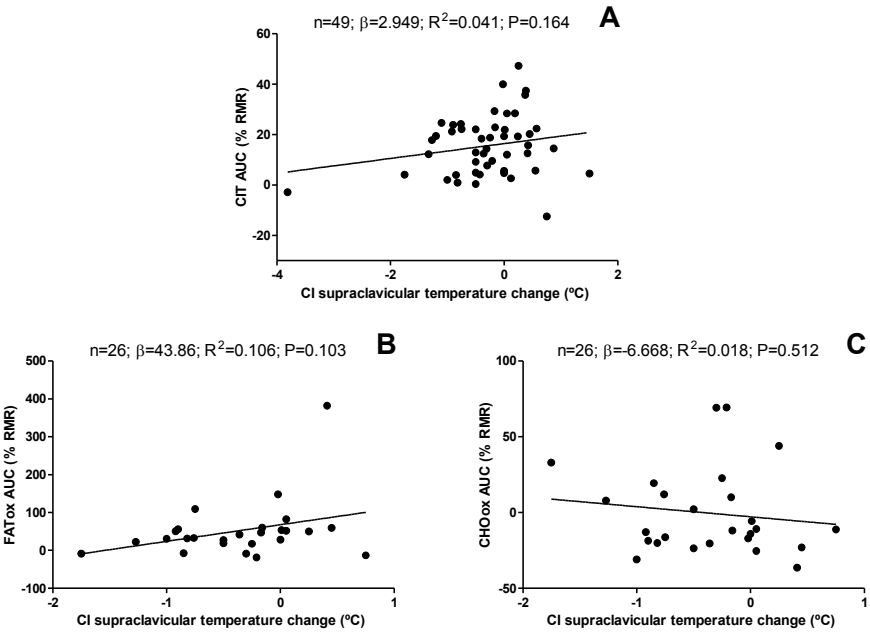

Figure 19. Association of cold-induced (CI) supraclavicular temperature change with cold induced thermogenesis (CIT) and cold-induced nutrient oxidation rates (including participants of studies 1 and 2). Unstandardized simple regression coefficient (β) and standardized coefficient of determination (R2). AUC: Area under the curve; RMR: resting metabolic rate; FATox: Fat oxidation rate; CHOox: Carbohydrates oxidation rate.

Finally, we also tested the above-mentioned associations using the difference between EE at the end of the cooling protocol and RMR, instead of the AUC calculation, and with % of EE coming from FATox instead of CI-NUTox. We found no significant associations in any of these analyses (data not shown).

DISCUSSION

This study analyzed the association of ^{18}F-FDG activity by BAT and skeletal muscle after a personalized cold exposure with CIT and CI-NUTox in young healthy adults. We also examined the association of supraclavicular skin temperature, an indirect marker of BAT activity [6,7], with CIT and CI-NUTox. No significant associations were found of BAT, skeletal muscle ^{18}F-FDG activity, or supraclavicular skin temperature with CIT and CI-NUTox. This lack of association was consistent across different methodologies for BAT and CIT assessment, and independent of several potential confounders. These findings are partially in line with other studies which used different methodologies [8–10], suggesting a negligible contribution of BAT to human CIT. On the other hand, the observed associations of skeletal muscle ^{18}F-FDG activity after a personalized cold exposure with CIT and CI-NUTox should be considered with caution, since not having ^{18}F-FDG activity in warm conditions might impair the ability to effectively assess cold-induced skeletal muscle metabolism.

The relation between BAT and CIT in humans has been extensively studied during the last years, yet, controversial results still exist. Several studies showed that individuals with detectable BAT (BAT+ in ^{18}F-FDG-PET-CT scan) present higher CIT levels [16,24,25], and that only BAT+ individuals present seasonal variation of CIT, being higher in winter than in summer [18]. Moreover, other studies showed positive and significant associations between BAT (assessed by ^{18}F-FDG PET-CT) and CIT [26–28]. In contrast, other studies did not observe any significant association between BAT and CIT [8,14,29], and BAT activation induced by cold acclimation was not accompanied by changes in CIT [30], which concur with our findings. Of note is that the lack of association observed in our study between CIT and supraclavicular skin temperature, as an indirect marker of BAT activity [6,7], further reinforces this finding.

Importantly, studies using $^{15}O[O_2]$, instead of ^{18}F-FDG, as the radiotracer for PET-CT scans, have demonstrated that the direct contribution of BAT to CIT is rather small (i.e. only 1% of the increase of CIT) [9,10]. Of note is that although $^{15}O[O_2]$ presents limitations due to a very short half-life (e.g. only a small anatomical area can be assessed) among others, it is able to effectively quantify EE of different tissues and not be affected by changes in substrate preference, as ^{18}F-FDG is. According to these studies, fully activated BAT in the cervical and upper thorax area (most human BAT) would account for only 10-15 Kcal/day if fully activated for 24 hours. However, paradoxically, some of the studies using radiotracers different from ^{18}F-FDG consistently showed a positive association of BAT perfusion and volume with CIT [9,31]. This, together with the observations showing higher glucose uptake and EE in skeletal muscles close to BAT depots [8,9], suggest that BAT may influence human CIT by indirect, rather than direct, mechanisms [9], such as endocrine signaling [32].

The hypothesis of an indirect effect of BAT over CIT could explain the controversy in the studies investigating the relation between BAT assessed by ^{18}F-FDG-PET-CT and CIT. Moreover, it is known that different methodological approaches for both PET-CT acquisition and analysis can profoundly influence the outcome [33,34]. Most studies examining the relation between BAT and CIT were conducted before a consensus was reached on how to perform PET-CT scans for BAT assessment and quantification [35], and thus, applied different methodologies. Therefore, methodological issues regarding cold

exposure prior to PET-CT and PET-CT analyses might explain the observed discrepancies. Here, we investigated the association between BAT and CIT in a larger sample size than previous studies, and we strictly followed current methodology for BAT assessment. However, it is to note that we measured BAT and CIT on different days, and, therefore, intra-individual day-to-day variance in EE may have prevented us from finding an existing association [36–39].

There is cumulating evidence supporting the idea that skeletal muscle is the main thermogenic organ upon cold exposure in humans [8,9,11,13], even at mild cold exposure. For instance, upon cold stimulation, EE of muscles in the cervical and upper thorax is ≈8 times higher than EE of BAT [9]. Interestingly, skeletal muscle contribution to CIT seems to be higher in deep and centrally located muscles than in superficial and bigger muscle groups [8,9]. Moreover, it is not clear whether the muscle EE during mild cold exposure relies upon shivering [8] or non-shivering mechanisms [13]. In contrast with this strong evidence, we found no association between skeletal muscle ^{18}F-FDG activity after a personalized cold exposure and CIT. However, it should be considered that we did not assess skeletal muscle ^{18}F-FDG activity in warm conditions, and, therefore, we could not determine whether the cold-induced change in glucose uptake was associated with CIT. This issue might not be of importance where BAT is concerned [35], since BAT glucose uptake in warm conditions is rather low [40]. However, differences between muscle ^{18}F-FDG activity in warm conditions and upon cold exposure are much smaller, and, therefore, not having skeletal muscle warm ^{18}F-FDG activity might have considerably limited the ability to detect an existing association. In addition to the skeletal muscle ^{18}F-FDG activity, we also tested the association between lean mass (as a subrogate of muscle mass) and CIT, which did not show significance (data not shown).

Moreover, it should be noted that skeletal muscle thermogenesis, even during shivering, relies mainly on fatty acid oxidation [41,42], and, therefore the glucose analog ^{18}F-FDG might not be a valid marker of muscle thermogenesis or metabolic activity. Similarly, BAT thermogenesis also relies mainly on FATox [4], and it has recently been shown that glucose uptake is not mandatory for human BAT thermogenesis [43]. Therefore, inherent limitations of ^{18}F-FDG for BAT detection and muscle activity quantification may explain why we failed to detect a physiologically plausible association. There is a need to develop new radiotracers for BAT detection and muscle activity quantification with more metabolic significance than ^{18}F-FDG and with a larger half-life than others, such as $^{15}O[O_2]$.

We also studied the associations of BAT and skeletal muscle ^{18}F-FDG activity with CI-NUTox. Since both BAT and skeletal muscle thermogenesis relies on fatty acid oxidation, it is plausible to expect a positive association of BAT and skeletal muscle activity with FATox. In contrast, we observed no association, which could be partially explained by the inherent limitations of ^{18}F-FDG as a radiotracer. However, since BAT and skeletal muscle thermogenesis seem to compensate each other [44] and both present a similar energy substrates consumption, it is also plausible that no relation with FATox exists. Finally, it should be considered that we recorded CI-NUTox in a cold exposure of only 1 hour. Longer cold exposures result in different contributions of both CHOox and FATox [41], and, therefore, new studies examining the relation of BAT and muscle thermogenesis with CI-NUTox during longer cold exposures are needed.

Our results should be considered with caution since some limitations are present. It should be noted that BAT and skeletal muscle ^{18}F-FDG activity was assessed on a different day than CIT and CI-NUTox, and, therefore, day-to-day variation may have influenced our results. Moreover, as stated above, whereas ^{18}F-FDG PET-CT after a personalized cold exposure is currently considered the gold standard for BAT in vivo quantification [35,45], it is not the best method to assess skeletal muscle metabolism upon cold exposure. Indeed, only having skeletal muscle ^{18}F-FDG activity at the end of cold exposure, and not having the uptake in warm conditions, largely impairs the capacity to assess cold-induced skeletal muscle ^{18}F-FDG activity, which could explain the lack of association with CIT. Moreover, we quantified skeletal muscle ^{18}F-FDG activity (SUVpeak) in one image slide, and therefore it might be influenced by the blood vessels eventually contained in the ROI. Using skeletal muscle SUVmean did not change the results, probably because muscle SUVmean and SUVpeak are highly correlated (all $r>0.976$; all $P<0.001$). Of note is also that the cooling protocol applied to assess both BAT and skeletal muscle ^{18}F-FDG activity and CIT and CI-NUTox is based on the individuals' shivering threshold, which was assessed by subjective methods (self-reported and direct observation), rather than by objective methods (electromyography). Another relevant issue is that the cold-exposure used to assess CIT and CI-NUTox was only one hour long, and, therefore, we cannot know whether a longer cold exposure would provide different results. In addition, it should be noted that we studied young healthy adults, hence we do not know whether these findings extend to older or unhealthy individuals. Finally, due to a lack of homogeneity in the fasting time of the ACTIBATE study, we conducted the NUTox analyses with a relatively small sample size (only 18 out of 44 participants in the ACTIBATE study). However, we performed a second study which allowed us to study the association of supraclavicular temperature with CIT and CI-NUTox in a larger sample size.

In conclusion, we found, in a larger sample size than previous studies and strictly following the most updated methodological recommendations, that BAT and skeletal muscle thermogenic activity (assessed by means of ^{18}F-FDG activity after a personalized cold exposure) is not associated with CIT or CI-NUTox. These findings support the hypothesis of BAT having a marginal role in human CIT, although important limitations inherent to the available technology for BAT and skeletal muscle metabolism in vivo quantification precludes us from drawing firm conclusions from the present data.

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Association of brown adipose tissue with resting and post prandial energy expenditure and nutrient oxidation rates

STUDY 5

BACKGROUND

BAT EE is increased by > 2-fold in response to cold exposure or after a mixed carbohydrate rich meal [1,2]. Nonetheless, considering the detected volume, the total contribution of BAT to whole-body EE was estimated to be 10±5 and 13±8 kcal/day in response to mild-cold [1] and a meal [2], respectively. On the other hand, Blondin et al. [3] showed that although the fractional post-prandial uptake of dietary fatty acids by human BAT is higher than in the muscle or white adipose tissue, the total contribution of BAT to post-prandial whole-body dietary fatty acid clearance is 0.3% approx.

The relation between BAT and cold-induced thermogenesis in humans have been largely studied during the last decade [1,4–12]. However, the relation of BAT with MIT and post-prandial nutrient oxidation rates (i.e. metabolic flexibility) has been much less studied and important gaps remain [13–18]. The nutrient meal composition is a key determinant of BAT post-prandial activity in murine, e.g. high-fat diet elicits a high BAT activity response [19,20]. Likewise, in humans, different meal challenges evokes markedly different postprandial nutrient oxidation rates [21]. Therefore, it is plausible that the contribution of BAT to human MIT and post-prandial metabolic flexibility depends on the meal composition and energy content, and thus the results of previous studies on the contribution of BAT to MIT and post-prandial nutrient oxidation may not be applicable to meals evoking a different post-prandial nutrient oxidation rate. On the other hand, it has recently been suggested that the association between BAT and MIT should be studied by means of heat production instead of EE, since most of the meal-induced EE correspond to the so-called obligatory part (i.e. the energy needed to digest, transport and store the nutrients) in which BAT is not expected to be involved [22].

In this study we investigated the associations of BAT volume and ^{18}F-FDG activity with basal and post-prandial EE and nutrient oxidation rates in young adults. Moreover, we also studied the association between BAT and meal-induced skin temperature changes.

METHODS OVERVIEW

General overview

A total of 112 (77 women) young adults (age 18-25 years) participated in the study (Table 15). Participants were enrolled in the ACTIBATE study.

Table 16 shows the methodology overview. For a more detailed methods description see “Methods” section.

Table 15. Descriptive characteristics of the participants.

	ALL			MEN			WOMEN		
	N	Mean	(SD)	N	Mean	(SD)	N	Mean	(SD)
Age (years)	112	22.13	(2.33)	35	22.42	(2.43)	77	22.00	(2.29)
BMI (kg/m^2)	112	24.49	(4.48)	35	26.30	(5.02)	77	23.68	(3.98)
Lean mass (kg)	104	41.28	(9.54)	33	52.35	(6.88)	71	36.13	(5.25)
Fat mass (kg)	104	24.05	(8.99)	33	23.56	(10.97)	71	24.28	(7.98)
Fat mass (%)	104	35.11	(7.86)	33	28.78	(7.38)	71	38.05	(6.19)
BMR (kcal/day)	112	1413.37	(319.96)	35	1654.65	(356.38)	77	1303.69	(231.73)
Basal fat oxidation (%EE)	92	37.98	(20.15)	29	39.90	(23.48)	63	37.10	(18.56)
Meal induced thermogenesis (%EI)	72	7.43	(9.06)	23	7.73	(9.60)	49	7.30	(8.89)
Delta RER (Max RER - Basal RER)	74	0.102	(0.073)	25	0.094	(0.103)	49	0.105	(0.052)
BAT volume (ml)	90	67.24	(55.53)	30	80.83	(67.87)	60	60.45	(47.40)
BAT metabolic activity	90	326.33	(313.59)	30	352.45	(356.09)	60	313.27	(292.39)
BAT SUV mean (g/ml)	90	3.80	(1.84)	30	3.39	(1.47)	60	4.00	(1.98)
BAT SUV peak (g/ml)	90	11.30	(8.16)	30	10.62	(7.90)	60	11.64	(8.34)
All muscles SUV peak (g/ml)	90	0.80	(0.20)	30	0.79	(0.18)	60	0.80	(0.21)
Deep muscles SUV peak (g/ml)	90	1.04	(0.30)	30	1.05	(0.29)	60	1.04	(0.31)
Cervical muscles SUV peak (g/ml)	90	1.07	(0.33)	30	1.04	(0.29)	60	1.08	(0.35)
Cold sensitive muscles SUV peak (g/ml)	90	0.92	(0.30)	30	0.90	(0.27)	60	0.92	(0.31)
Descending aorta SUV peak (g/ml)	90	1.52	(0.35)	30	1.63	(0.41)	60	1.46	(0.31)

Data are presented as means (standard deviation). BMI: Body mass index; BMR: Basal metabolic rate; EE: Energy expenditure; EI: Energy intake; RER: Respiratory exchange ratio; Max: Maximum; BAT: Brown adipose tissue; SUV: Standardized uptake value.

Statistical analysis

Data are expressed as mean±SD, otherwise stated. We conducted linear regression analyses to analyze the association of BAT and skeletal muscle ^{18}F-FDG activity related parameters with BMR, resting FATox, MIT, and the deltas of RER, CHOox and FATox. We repeated the analyses after including potential confounders in the model such as date when the PET-CT was performed, sex, and BMI. We also used the same regression models to study the associations of basal and post-prandial supraclavicular skin temperature with BMR, basal FATox, MIT, and the deltas of RER, CHOox and FATox. We also conducted regression analyses to study the associations of the BAT and skeletal muscle ^{18}F-FDG activity related parameters with meal-induced changes in skin temperature (mean, proximal, distal and supraclavicular). We used one-factor ANOVA to study the dynamics of EE, RER, CHOox and FATox, using the Bonferroni correction in post-hoc comparisons. Moreover, we used a two-factor (Time*PET variable) ANOVA to check for the interaction of the BAT and skeletal muscle ^{18}F-FDG activity related parameters with changes on time of

EE, RER, CHOox and FATox. 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.

Table 16. Study 5 methodology.

GENERAL INFORMATION	
General aim	To study the association of BAT and skeletal muscle ^{18}F-FDG activity with BMR and MIT
Design	Cross-sectional
Cohort and participants	ACTIBATE (n=112)
OUTCOMES INCLUDED IN THE STUDY	
Outcome	Variables included
PET-CT	BAT volume (-190/-10 HU; Ind. SUV threshold.) BAT metabolic activity (-190/-10 HU; Ind. SUV threshold.) BAT SUVmean (-190/-10 HU; Ind. SUV threshold.) BAT SUVpeak (-190/-10 HU; Ind. SUV threshold.) All muscles SUVpeak Deep muscles SUVpeak Cervical muscles SUVpeak Cold sensitive muscles SUVpeak
BIC	BMR Basal FATox
PPIC	MIT Meal-induced Δ RER (Max-basal) Meal-induced Δ RER (Min-basal) Meal-induced Δ CHOox (Max-basal) Meal-induced Δ CHOox (Min-basal) Meal-induced Δ FATox (Max-basal) Meal-induced Δ FATox (Min-basal)
SkTa	Basal mean skin temperature Basal proximal skin temperatura Basal distal skin temperature Basal supraclavicular skin temperature Meal-induced mean skin temperature Meal-induced proximal skin temperature Meal-induced distal skin temperature Meal-induced supraclavicular skin temperature

BAT: Brown adipose tissue; ^{18}F-FDG: ^{18}F-Fluorodeoxuglucose; PET-CT: Positron emission tomography-Computerized tomography; Ind: Individualized; SUV: Standardized uptake value; BIC: Basal indirect calorimetry; BMR: Basal metabolic rate; FATox: Fat oxidation; PPIC: Post-prandial indirect calorimetry; MIT: Meal-induced thermogenesis; CHOox: carbohydrates oxidation; SkTa: Skin temperature assessment.

RESULTS

There were no significant associations (All P>0.15) of BAT and skeletal muscle ^{18}F-FDG activity related parameters with BMR (Figure 20) and basal FATox (Figure 21).

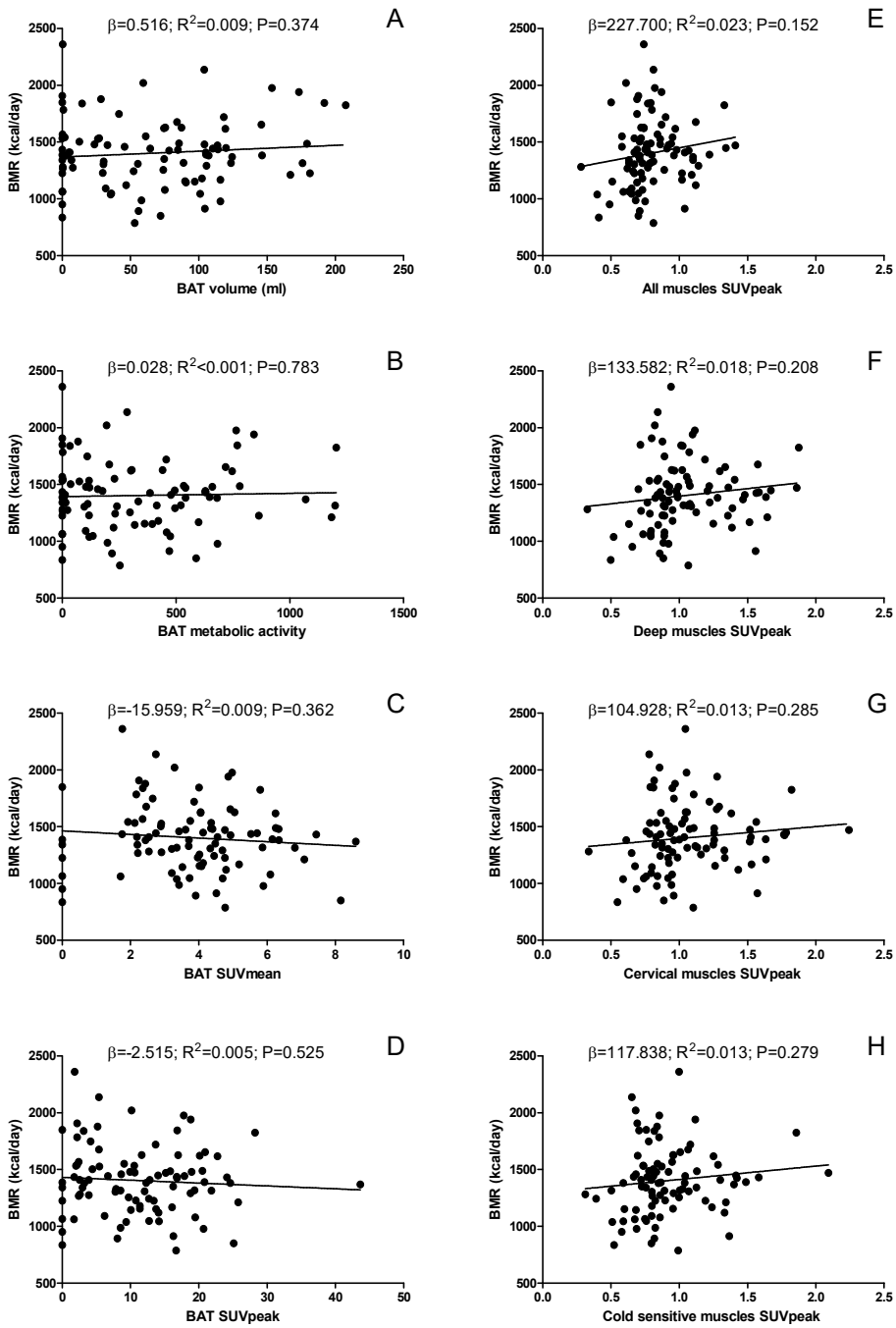

Figure 20. Associations of basal metabolic rate (BMR) with brown adipose tissue (BAT) and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation. Unstandardized β, R2, and P from simple linear regressions. SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

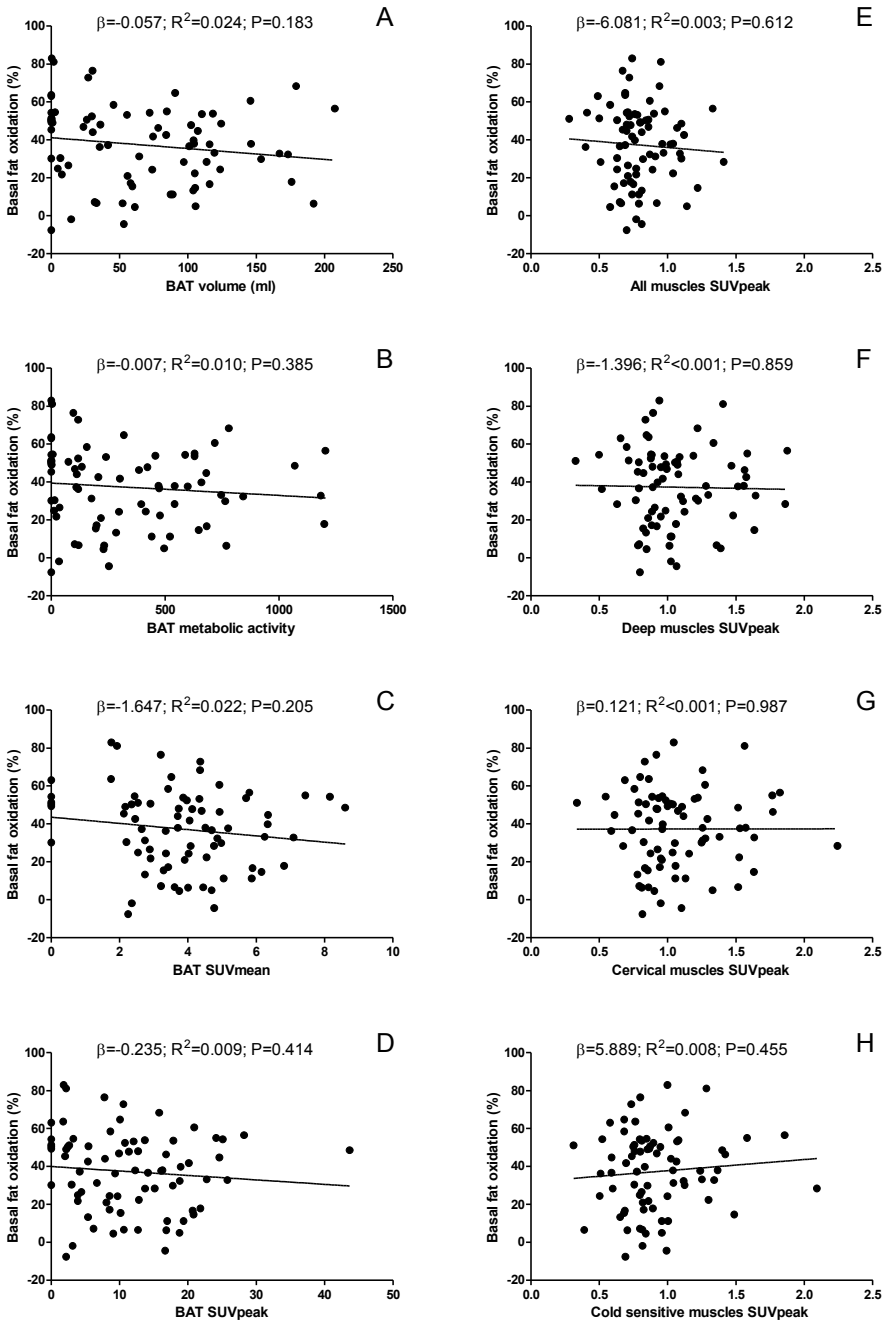

Figure 21. Associations of basal fat oxidation (% of energy expenditure) with brown adipose tissue (BAT) and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation. Unstandardized β, R2, and P from simple linear regressions. SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

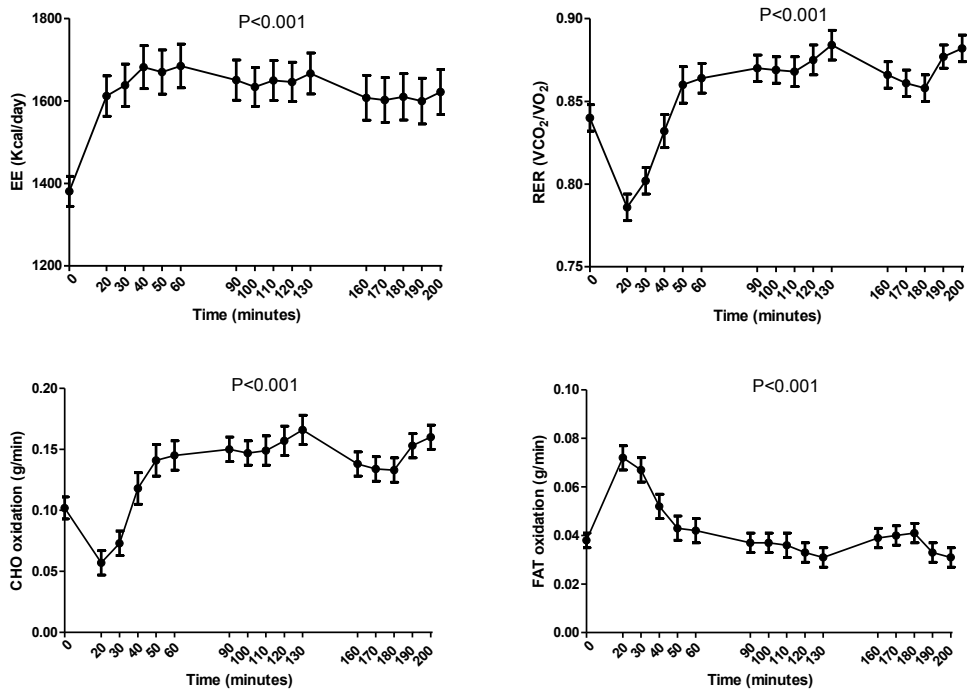

Figure 22. Energy expenditure (EE), respiratory exchange ratio (RER), carbohydrates (CHOox) and fat oxidation (FATox) in response to a mixed meal liquid ingestion. VCO2: carbon dioxide production; VO2: oxygen consumption; min: minutes. P for one-way analysis of variance.

Figure 22 shows the dynamics of EE, RER, CHOox and FATox during the post-prandial period. All parameters changed over time (all P<0.001). There was no interaction effect of BAT and skeletal muscle ^{18}F-FDG activity related parameters with dynamics of EE, RER, CHOox and FATox (All P>0.15; Table 17), except for the interaction between the RER dynamic and BAT SUVmean (P=0.046; Table 17). There was no significant association between BAT metabolic activity, SUVmean and SUVpeak, and skeletal muscle ^{18}F-FDG activity related parameters with MIT (Figure 23, all P>0.12), which persisted after controlling for potential confounders (data not shown). BAT volume was weakly associated with MIT (β=0.031; R^2=0.056; P=0.071), although the association disappeared after adjusting by the date when the PET-CT was performed, sex and BMI (P=0.222, data not shown). Since we observed a different nutrient oxidation rate pattern in the first 30 minutes of the post-prandial period (see Figure 22) we also tested the association of BAT and skeletal muscle ^{18}F-FDG activity related parameters with the increase in EE 30 minutes after the meal ingestion. BAT volume was positively associated with MIT during the first 30 minutes (β=0.004; R^2=0.069; P=0.041), although the associations were attenuated after adjusting by the date when the PET-CT was performed, sex and BMI (P=0.180). We observed no association of the rest of BAT and skeletal muscle ^{18}F-FDG activity related parameters with MIT during the first 30 minutes (All P>0.130, data not shown). In addition, BAT mean radiodensity was not associated to MIT (All P>0.438, data not shown).

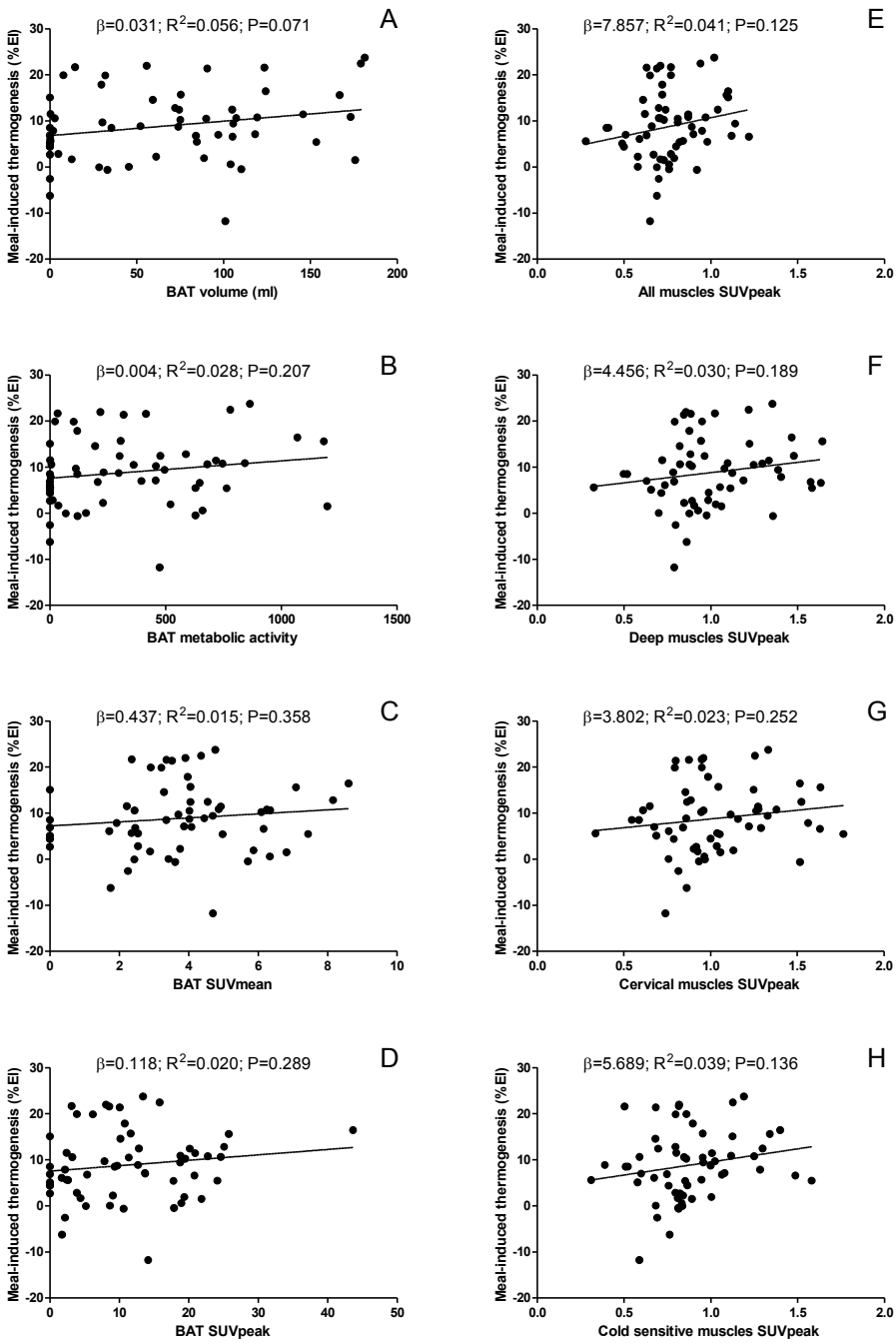

Figure 23. Associations of meal induced thermogenesis (% of energy intake) with brown adipose tissue (BAT) and skeletal muscle cold-induced ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation. Unstandardized β, R2, and P from simple linear regressions. SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

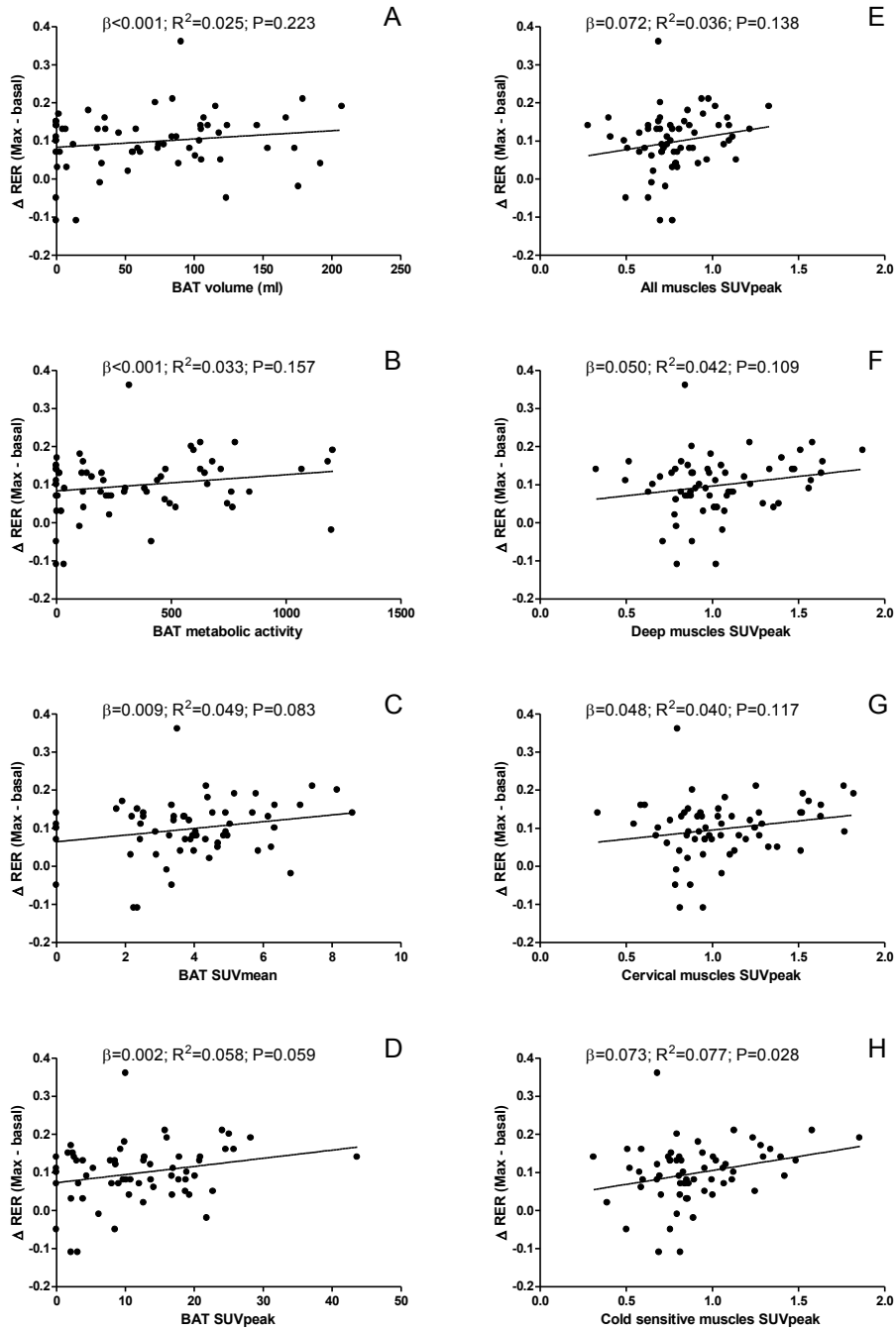

Figure 24. Associations of meal induced delta in respiratory exchange ratio (RER) with brown adipose tissue (BAT) and skeletal muscle cold-induced ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation. Unstandardized β, R2, and P from simple linear regressions. SUV: Standardized uptake value; Max: maximum; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

Figure 24 shows the association of BAT and skeletal muscle ^{18}F-FDG activity related parameters with post-prandial metabolic flexibility. BAT SUVmean (P=0.083), SUVpeak (P=0.059), and cold sensitive muscles SUVpeak (P=0.028) were associated with the difference between the maximum RER and the basal RER, yet, associations of SUVmean and SUVpeak were attenuated after controlling for the date when the PET-CT was performed, sex, and BMI. In contrast, the association of cold sensitive muscles SUVpeak with the difference between the maximum RER and the basal RER in the multiple regression models persisted (All P<0.076; data not shown). We further explored the association of cold sensitive muscles SUVpeak and the post-prandial metabolic flexibility (Figure 25) and observed that cold sensitive muscles SUVpeak is positively associated with the post-prandial delta in CHOox (P=0.007), which remained after controlling for the date when the PET-CT was performed, sex, and BMI (All P<0.032). No associations were however observed between cold sensitive muscles SUVpeak and delta in FATox (P=0.751). We observed no significant associations of BAT and skeletal muscle ^{18}F-FDG activity with the rest of metabolic flexibility deltas (data not shown). BAT mean radiodensity was no associated with any of the metabolic flexibility indicators (All P>0.434, data not shown).

Table 17. Interaction effect of brown adipose tissue (BAT) and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation with energy expenditure (EE), respiratory exchange ratio (RER), carbohydrates (CHOox) and fat oxidation (FATox).

	EE	RER	CHOox	FATox
	P for interaction	P for interaction	P for interaction	P for interaction
BAT volume (ml)	0.553	0.447	0.176	0.375
BAT metabolic activity	0.820	0.502	0.346	0.528
BAT SUVmean (g/ml)	0.939	0.046	0.138	0.259
BAT SUVpeak (g/ml)	0.897	0.204	0.194	0.356
All muscles SUVpeak (g/ml)	0.241	0.715	0.421	0.563
Deep muscles SUVpeak (g/ml)	0.165	0.665	0.373	0.579
Cervical muscles SUVpeak (g/ml)	0.262	0.646	0.402	0.667
Cold sensitive muscles SUVpeak (g/ml)	0.386	0.441	0.306	0.380

P from a one-factor mixed ANCOVA (BAT/Muscle ^{18}F-FDG activity as covariable) applying Greenhouse Geiser correction. SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

There was no significant associations of basal supraclavicular skin temperature and meal-induced change in supraclavicular skin temperature with BMR, basal FATox, MIT and the delta between the maximum RER and the basal RER (all P>0.19; Figure 26). Finally, none of the BAT and skeletal muscle ^{18}F-FDG activity related parameters were associated (All P>0.28) with the meal-induced changes in mean (Figure 27), proximal (Figure 28), distal (Figure 29) and supraclavicular (Figure 30) skin temperature.

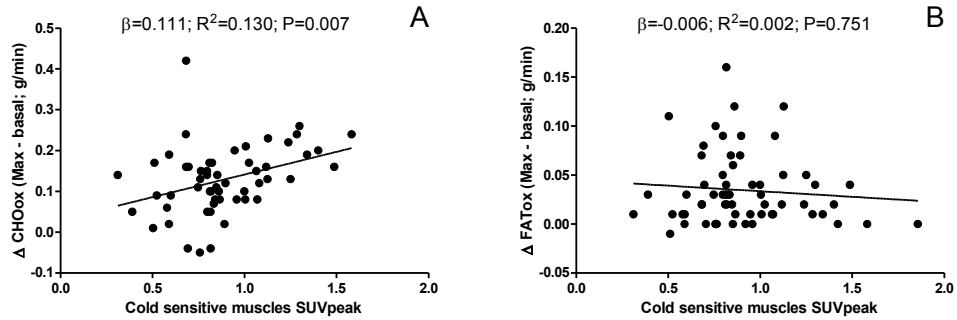

Figure 25. Associations of meal induced delta in nutrient oxidation rate with cold-sensitive skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation. Unstandardized β, R2, and P from simple linear regressions. SUV: Standardized uptake value; Max: maximum; CHOox: Carbohydrates oxidation; FATox: Fat oxidation; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

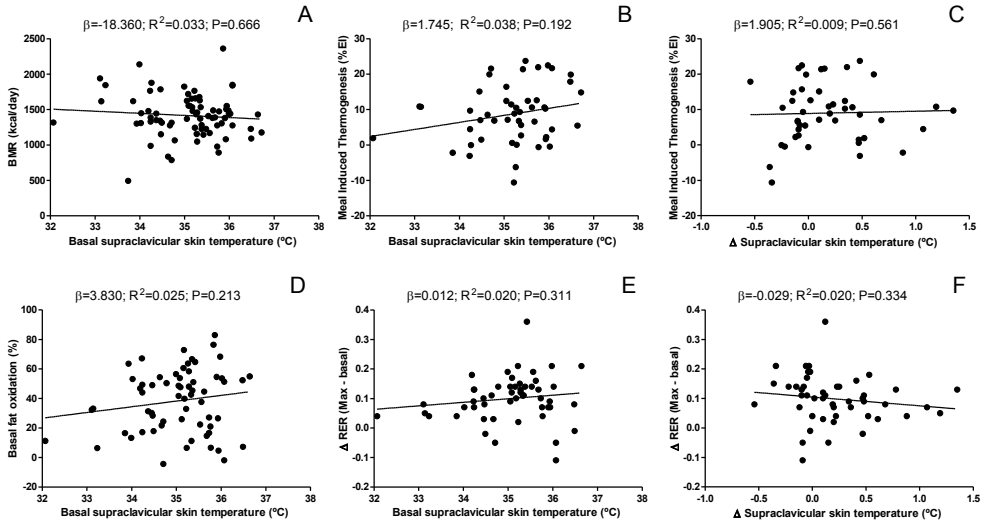

Figure 26. Associations of basal metabolic rate (BMR), basal fat oxidation, meal-induced thermogenesis and meal-induced delta in respiratory exchange ratio (RER), with basal supraclavicular skin temperature and meal-induced delta in supraclavicular skin temperature. Unstandardized β, R2, and P from simple linear regressions. EI: energy intake; Max: maximum.

DISCUSSION

The results of this study show that BAT is not associated to basal and post-prandial EE and nutrient oxidation rates in humans. Likewise, we observed that BAT is not associated to meal-induced changes in mean, proximal, distal and supraclavicular skin temperature. These findings concur with previous studies[1–3,17] and provide further evidence indicating that BAT plays a negligible role on basal and post-prandial EE in humans. We observed however that the ^{18}F-FDG activity after an individualized cold stimulation in cold sensitive muscles [1,4] seems to be associated with post-prandial increase in CHOox, a sign of metabolic flexibility in response to a mixed-composed meal.

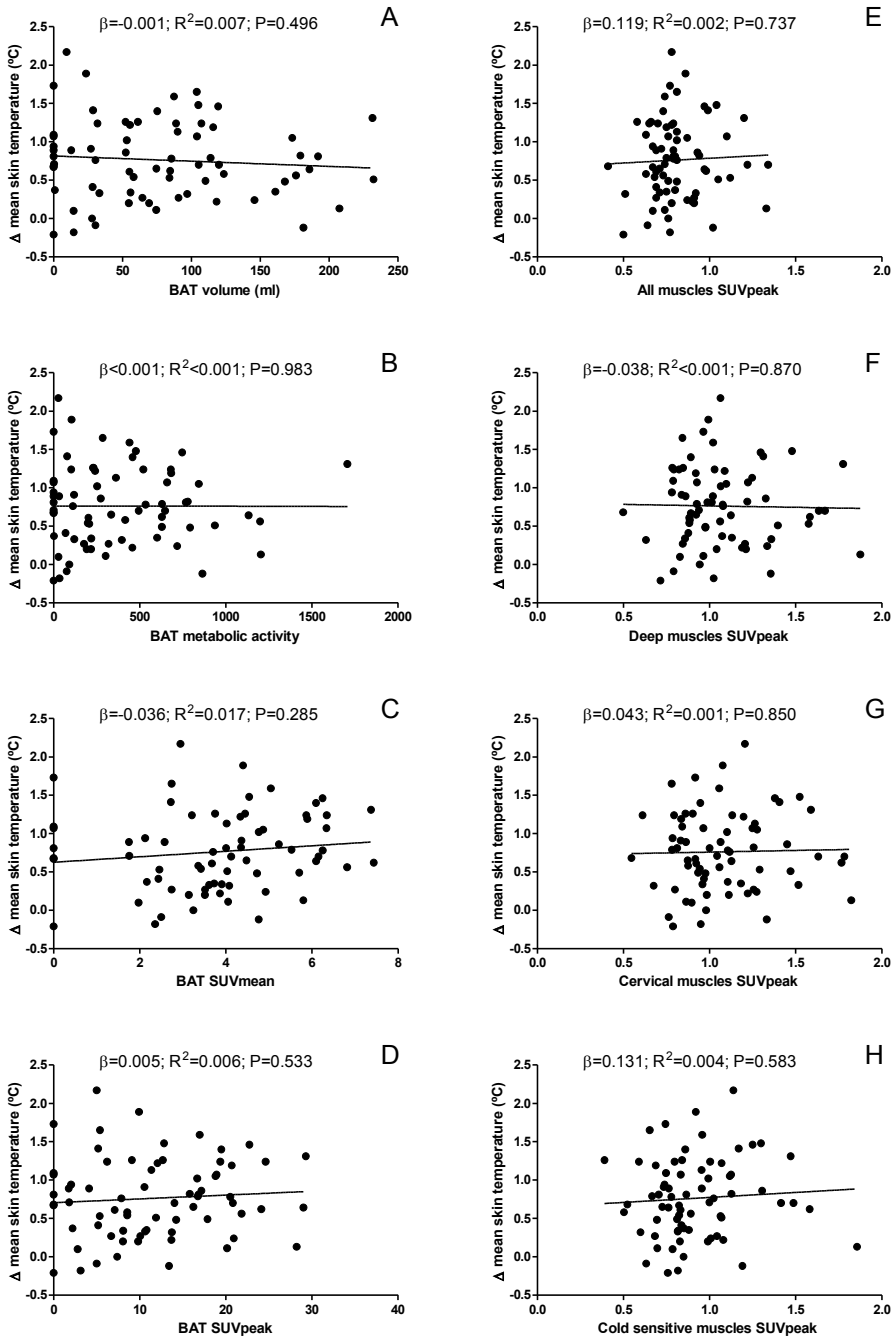

Figure 27. Associations of meal induced delta in mean skin temperature with brown adipose tissue (BAT) and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation. Unstandardized β, R2, and P from simple linear regressions. SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

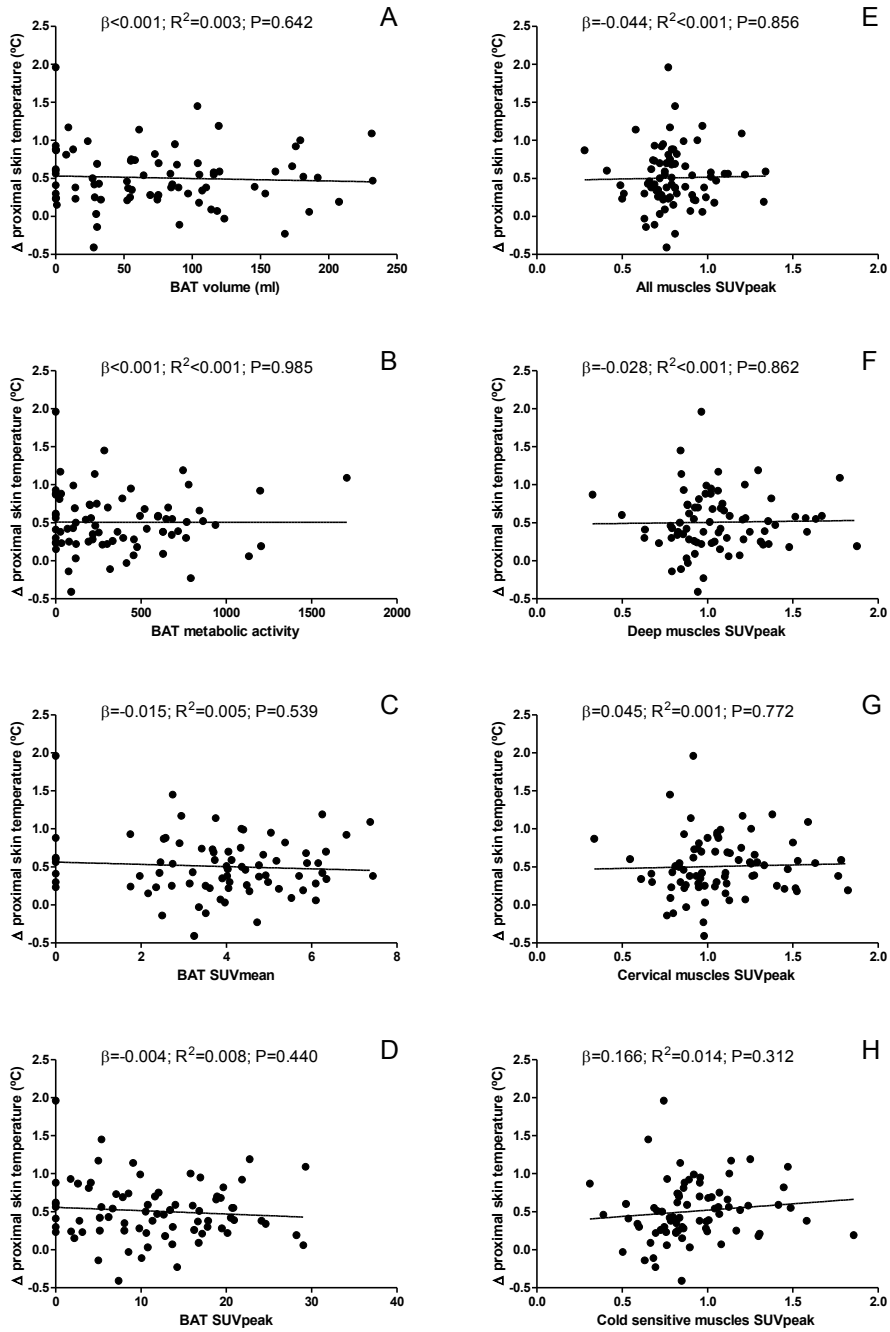

Figure 28. Associations of meal induced delta in proximal skin temperature with brown adipose tissue (BAT) and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation. Unstandardized β, R2, and P from simple linear regressions. SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

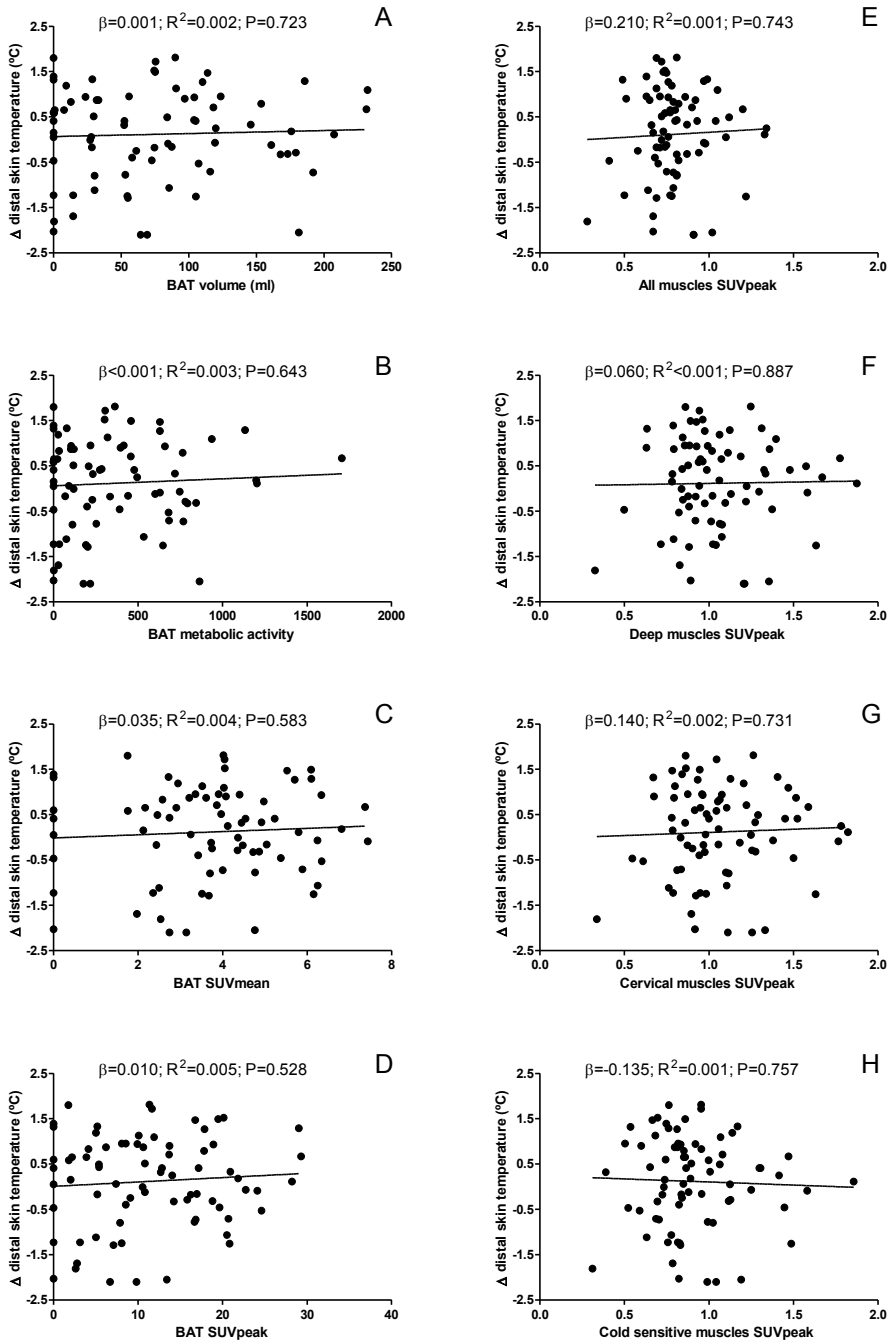

Figure 29. Associations of meal induced delta in distal skin temperature with brown adipose tissue (BAT) and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation. Unstandardized β, R2, and P from simple linear regressions. SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

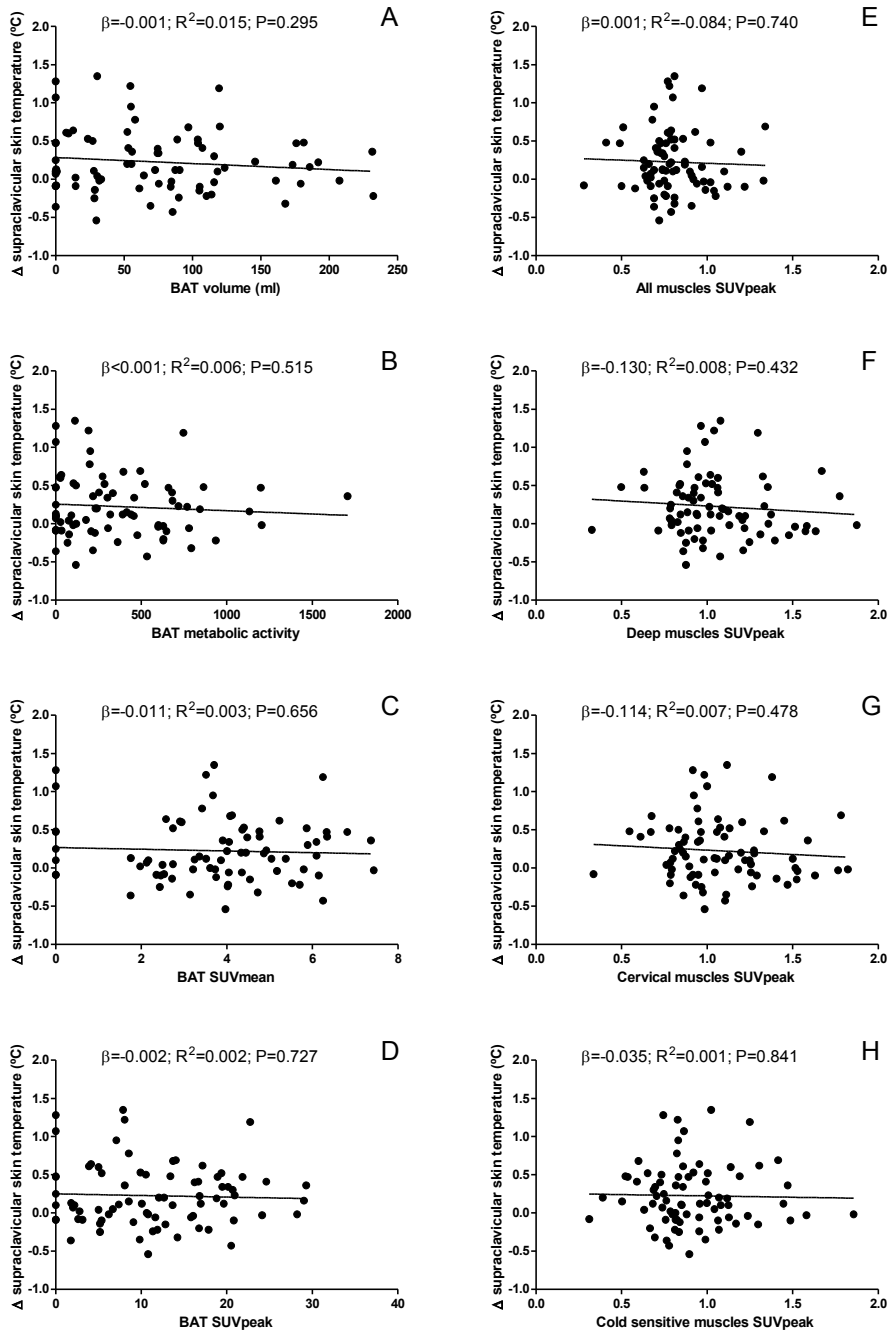

Figure 30. Associations of meal induced delta in supraclavicular skin temperature with brown adipose tissue (BAT) and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation. Unstandardized β, R2, and P from simple linear regressions. SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

BAT is not associated with basal energy expenditure and nutrient oxidation rates

The main function of BAT is to defend core body temperature from cooling. Therefore, BAT activity is increased by cold exposure, but its activity remains relatively low at thermoneutrality [1]. We measured BMR in a warm environment (22-24°C) and while participants were covered by a sheet following the recommendations for assessing thermoneutral BMR [23]. Therefore, even assuming that BAT can significantly contribute to human EE [24,25], it is expected not to find an association with BMR and basal FATox, which was confirmed by our findings.

First human BAT studies speculated with the possibility of BAT influencing BMR at ambient temperature [26,27]. This is the case for rodents, whose thermoneutral temperature range is usually above the ambient temperature [28]. Moreover, previous studies have reported an association between BAT and BMR [8,26] or whole-day FATox [14] in humans. However, methodological issues related to BAT assessment by PET-CT [29] and/or pooling data of different cohorts [8] plausibly biased these associations.

BAT is not associated with meal-induced thermogenesis

Diet induced thermogenesis is defined as the increase in EE required for the digestion, absorption and storage of food energy throughout a day [30]. Even a single meal is known to induce an increase in thermogenesis, which is known as MIT. MIT is typically divided into an obligatory and a facultative part [31]. While the obligatory component is the energy needed for the digestion, absorption, distribution and storage of nutrients [32], the facultative component is the energy expended above the calculated requirement for the obligatory part [32–34]. Currently, it is not known which functions are responsible of the facultative component EE [32–34]. Both BAT activity and the facultative part of MIT are known to be mediated by SNS activity, and therefore it is biological plausible that BAT derived EE accounts for part of the facultative component of MIT.

U Din et al. [2] recently showed that the energy consumption of BAT during the post-prandial period after a mixed-composed meal is increased in a similar extend that in response to mild cold exposure, and is ≥2 times higher than the energy consumption in warm situations. However, the total contribution of BAT EE to whole-body EE was shown to be very weak. This negligible direct contribution to whole-body EE is similar in CIT [1,4–6]. However, it has been proposed that BAT may orchestrate NST in other tissues, such as skeletal muscle, by endocrine mechanisms [1,35], which would explain the commonly found association between BAT and CIT. Further evidence is needed to confirm this hypothesis. Nonetheless, if BAT activity is quantitatively similar upon cold exposure and after a meal, it is tempting to speculate with the possibility of this hypothetical indirect BAT contribution to EE being also present in post-prandial situations. If it was the case, BAT related parameters should be associated to MIT.

The association between human BAT and MIT is controversial [14–17,36]. For instance, Vosselman et al. showed an increase in BAT ^{18}F-FDG activity after a high-fat meal, although no association between BAT activity and MIT was found [13]. Hibi et al. [14] found higher MIT under thermoneutral conditions in a group of PET+ (i.e. detectable BAT in ^{18}F-FDG-PET/CT) than in a group of PET- (i.e. no detectable BAT in ^{18}F-FDG-PET/CT). Wijers et al. [15] found that although EE responses to mild cold exposure and overfeeding were highly

variable between individuals, both phenomenon were highly correlated, being norepinephrine plasma concentrations significantly correlated to EE increase in both situations. On the other hand, there are several studies suggesting that BAT is not associated with MIT in humans. Peterson et al. [16] reported no association between MIT response to overfeeding and CIT thermogenesis, neither changes on MIT response to overfeeding after a cold acclimation period. These results concur with those by Schlögl et al. [18] who found a decrease in BAT ^{18}F-FDG activity after 24 hours overfeeding period. In conclusion, the relation between BAT, overfeeding and MIT in humans is still controversial, and further research is needed to bring light to this issue. We found, in a larger sample size than previous studies, that BAT volume and ^{18}F-FDG activity, assessed strictly following current methodological recommendations [29], are not associated with MIT in young adults after adjusting by some important confounders (the date when the PET-CT was performed, sex and BMI). This suggest that the above mentioned hypothetical indirect action of BAT on whole-body EE after a meal does not seem to be true.

Recently, it has been suggested that BAT role in the post-prandial period should be focused on heat generation, rather than on EE, since changes in the obligatory part may bias the relation between BAT and thermogenesis [22]. We recently showed that skin temperature is modified during the post-prandial period suggesting an increase in heat production (i.e. thermogenesis) [37]. Importantly, we have observed that the supraclavicular skin temperature, considered a proxy of BAT heat production [38–40], was not associated with MIT. Moreover, we observed that the BAT related parameters were not associated with the meal-induced changes in mean, proximal, distal and supraclavicular skin temperature. These findings concurs with another study [17], showing that BAT thermal activity does not change after 8 weeks of overfeeding. Taken together, these results suggest that BAT is not significantly contributing to human heat production during the post-prandial period.

U Din et al. studies showed that skeletal muscle seems to be the main contributor to NST, and that skeletal muscle EE is similar upon cold exposure than after a meal challenge, despite blood flow upregulation seems to be lower in the post-prandial state [1,2]. Here we found that skeletal muscle ^{18}F-FDG activity after an individualized cold exposure is not associated with meal-induced EE or skin temperature changes. It should be noted however that these radiological parameters are not good markers of muscle thermogenesis. We did not assess skeletal muscle ^{18}F-FDG activity in warm conditions, and, therefore, we could not determine whether the cold-induced change in glucose uptake was associated with MIT. Moreover, it is clear that glucose uptake and thermogenesis are not necessarily related, especially in conditions of hyperinsulinemia, such as in the post-prandial state [41].

BAT is not associated with post-prandial metabolic flexibility

Metabolic flexibility is defined as the adaptive response of an organism's metabolism to maintain energy homeostasis by matching fuel availability and demand to periodic fasting, varying meal composition, PA, and environmental fluctuation [21]. Importantly, obesity and other metabolic diseases are associated with metabolic inflexibility during the post-prandial period, and metabolic flexibility is thought to importantly influence the metabolic health of humans [42]. The whole-body post-prandial metabolic flexibility results

from the coordinated action of several mechanism (e.g. increasing glucose uptake in muscle, reducing fatty acid release by adipocytes and increase its triglyceride uptake, etc.), among which BAT nutrients uptake could play a role. A mixed-composed carbohydrate rich meal like the one used in our study typically produces an increase in RER, corresponding to an increase in CHOox. However, we observed an increased FATox rate after the meal. It should be considered that the used shake contains relevant amount of medium-chain fatty acids and taurine (http://vegenatnutricion.es/index.php?r=nutricion/producto&id=10), which have been proven to increase FATox [43,44]. Moreover, we provided the shake at a temperature of 4°C, which is able to reduce core body temperature [45], that elicit a thermogenic response mainly dependent of FATox [46]. Therefore, the post-prandial decrease in RER observed in our study could be due to the composition of the shake, to its temperature or even a combined effect of both factors. Nonetheless, this decrease in RER suggest that this type of meal might be an optimal meal challenge to study BAT contributions, since BAT metabolism mainly relates on FATox [47,48].

In mice, BAT clearly contributes to post-prandial nutrient clearance, functioning like a sink, which prevents the rodent from hyperglycemia and hypertriglyceridemia [49]. In humans however there is convincing evidence of a marginal role of BAT in post-prandial nutrient clearance, even if it is concomitantly stimulated by cold [3]. This negligible role is also supported by calculations made with bioenergetic data obtained from human studies [4,6,41]. Therefore, our results showing a lack of association between BAT related parameters and post-prandial metabolic flexibility are in agreement with previous studies, and also points to a marginal role of BAT in human energy homeostasis regulation.

Of note, we detected a significant association between cold sensitive muscles ^{18}F-FDG activity after an individualized cold stimulation and post-prandial metabolic flexibility. Previous studies have shown that there are several deep-located skeletal muscle groups that exhibits a marked increase in metabolism in response to cold exposure [1,4,50]. Whether this augmented metabolism corresponds to shivering or non-shivering mechanism remains to be elucidated [4,51]. Interestingly, U Din et al. [2] showed that these muscle groups also present an augmented metabolism in post-prandial situations. Our findings suggest that these muscles group metabolism could play an important role, not only in EE, but also in nutrient handling after a meal. More studies are needed to elucidate whether there is a causal relation explaining this association.

Some limitatons should be considered when interpreting these results. It should be noted that the observational design of our study precludes to stablish causal relationships. We performed the PET-CT for BAT and muscle ^{18}F-FDG activity assessment in a different day than the EE evaluation. Therefore, intra-individual day-to-day variability might have contributed to the lack of association. Moreover, we performed the PET-CT after an individualized cold-exposure, which is currently considered the best available technique to assess and quantify human BAT volume and ^{18}F-FDG activity in vivo [29,52]. It might not be however a valid technique to infer BAT and skeletal muscle activity during the post-prandial period. Finally, currently commercially available metabolic carts present significant measurement error [53], specially for post-prandial assessment [54], and thus it may also preclude us from finding a really occurring association.

This study shows that BAT volume and ^{18}F-FDG activity after an individualized cold exposure are not associated with basal and post-prandial EE and nutrient oxidation rates in young adults. We observed however that the ^{18}F-FDG activity of some deeply located skeletal muscles after an individualized cold exposure is positively associated with a marker of post-prandial metabolic flexibility, which may suggest that these muscle groups are not only playing a relevant role in CIT, but also in post-prandial nutrient handling.

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Association of brown adipose tissue with energy intake and appetite regulation

STUDY 6

BACKGROUND

It was hypothesized that BAT could significantly contribute to human EE [1–3]. Indeed, initial studies showed positive associations between BAT and NST in humans [4–7]. Importantly, it should be noted that EE is a potent driver of energy intake [8–11], and therefore, even if BAT significantly contributes to human EE, its possible role could be to increase energy intake rather than creating a negative energy balance through raised EE. These issues suggest that the link between BAT and appetite regulation should be more rigorously examined to fully understand the contribution of BAT to human energy balance [12].

The relationship between BAT and energy intake has been previously addressed [11,13]. In murine models, endocrine mechanisms connecting BAT activity to energy intake regulation are already known [14,15]. However, under physiological conditions, it has been demonstrated that BAT recruitment by means of cold exposure is coupled with an increase in energy intake, resulting in no change in the animal´s body composition [11]. Importantly, human BAT volume has been shown to be positively correlated with fasting and cold-induced concentrations of some peptides involved in appetite regulation, suggesting a potential cross talk between BAT and the enteropancreatic axis, which points to a possible role of BAT in human appetite regulation [13]. However, energy intake regulation results from a complex interaction of biological and psychological processes [9], and therefore, there is a need to directly study the relationship between BAT, appetite and energy intake.

This study aimed to investigate the association of BAT volume and BAT and skeletal muscle ^{18}F-FDG activity after an individualized cold stimulation, with energy intake and appetite-related sensations in young healthy humans.

METHODS OVERVIEW

General overview

A total of 148 participants (99 women) took part in the study (Table 18). Participants were part of the ACTIBATE study [16].

Table 19 shows the methodology overview. For a more detailed methods description see “Methods” section.

Statistical analyses

Descriptive statistics are presented as mean ± SD, unless otherwise stated. We used simple linear regression analyses to test the association of BAT and skeletal muscle ^{18}F-FDG activity, and body composition variables, with energy intake. We also used multiple linear regression models to test the associations of BAT volume and BAT and skeletal muscle ^{18}F-FDG activity after an individualized cold stimulation adjusting by the date when the PET-CT was performed (Model 1), Model 1 plus sex (Model 2), and model 2 plus fat mass and lean mass (Model 3). On the other hand, we use repeated-measures ANCOVA to study the interaction of BAT volume and BAT and skeletal muscle ^{18}F-FDG activity after an individualized cold stimulation with time in the appetite related sensations. Furthermore, we divided the participants in tertiles of BAT-related variables

and skeletal muscle glucose uptake, and later compared the kinetics of appetite-related sensations on time between the highest and the lowest BAT and skeletal muscle glucose uptake tertile with a two factor (Time*Group) ANOVA. Finally, we used simple linear regression and the Bland-and-Altman method [17] to study the congruence between the two methods of energy intake assessment (ad-libitum meal and dietary recalls).

The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 21.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set at <0.05.

Table 18. Characteristics of the study participants.

	ALL			MEN			WOMEN		
	N	Mean	(SD)	N	Mean	(SD)	N	Mean	(SD)
Age (years)	148	22.12	(2.21)	49	22.35	(2.29)	99	22.00	(2.17)
BMI (kg/m^2)	148	24.90	(4.63)	49	26.92	(5.38)	99	23.90	(3.87)
Lean mass (Kg)	141	41.40	(9.63)	46	52.55	(7.11)	95	36.00	(4.87)
Fat mass (Kg)	141	24.97	(8.72)	46	25.31	(10.98)	95	24.81	(7.45)
Fat mass (%)	141	35.96	(7.55)	46	30.24	(7.52)	95	38.73	(5.82)
LMI (kg/m^2)	141	14.57	(2.38)	46	17.08	(2.05)	95	13.36	(1.35)
FMI (kg/m^2)	141	8.89	(3.00)	46	8.24	(3.57)	95	9.21	(2.65)
VAT (g)	141	336.04	(175.40)	46	421.39	(174.91)	95	294.71	(160.89)
BAT volume (ml)	120	71.41	(59.76)	40	84.20	(71.19)	80	65.02	(52.47)
BAT metabolic activity	120	353.74	(346.65)	40	364.98	(368.79)	80	348.12	(337.30)
BAT SUVmean (g/ml)	120	3.89	(1.87)	40	3.41	(1.40)	80	4.13	(2.04)
BAT SUVpeak (g/ml)	120	11.64	(8.33)	40	10.47	(7.74)	80	12.23	(8.59)
All muscles SUVpeak (g/ml)	120	0.81	(0.19)	40	0.80	(0.17)	80	0.81	(0.20)
Deep muscles SUVpeak (g/ml)	120	1.06	(0.29)	40	1.05	(0.27)	80	1.06	(0.30)
Cervical muscles SUVpeak (g/ml)	120	1.07	(0.31)	40	1.04	(0.27)	80	1.08	(0.33)
Cold sensitive muscles SUVpeak (g/ml)	120	0.92	(0.28)	40	0.88	(0.25)	80	0.93	(0.30)
Descending aorta SUVpeak (g/ml)	120	1.57	(0.34)	40	1.66	(0.37)	80	1.52	(0.32)
Ad libitum meal: energy consumed (kcal)	128	899.69	(398.21)	45	1193.97	(457.20)	83	740.14	(245.22)
Ad libitum meal: rate of eating (kcal/min)	124	77.75	(38.54)	43	107.02	(44.87)	81	62.20	(22.83)

BMI: Body mass index; LMI: Lean mass index; FMI: Fat mass index; VAT: Visceral adipose tissue; BAT: Brown adipose tissue; SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

Table 19. Study 6 methodology.

GENERAL INFORMATION	
General aim	To study the association of BAT and skeletal muscle ^{18}F-FDG act. with APP
Design	Cross-sectional
Cohort and participants	ACTIBATE (n=144)
OUTCOMES INCLUDED IN THE STUDY	
Outcome	Variables included
PET-CT	BAT volume (-190/-10 HU; Ind. SUV threshold.) BAT metabolic activity (-190/-10 HU; Ind. SUV threshold.) BAT SUVmean (-190/-10 HU; Ind. SUV threshold.) BAT SUVpeak (-190/-10 HU; Ind. SUV threshold.) All muscles SUVpeak Deep muscles SUVpeak Cervical muscles SUVpeak Cold sensitive muscles SUVpeak
APP	Ad libitum energy intake Energy intake estimated by 24 hours dietary recalls Hungry sensation Fullness sensation
BCa	BMI Lean mass LMI Fat mass Fat mass percentage FMI

BAT: Brown adipose tissue; ^{18}F-FDG: ^{18}F-Fluorodeoxyglucose; APP: Energy intake and appetite regulation; PET-CT: Positron emission tomography-Computerized tomography; Ind: Individualized; SUV: Standardized uptake value; BCa: Body composition assessment; BMI: Body mass index; LMI: Lean mass index; FMI: Fat mass index.

RESULTS

There were no associations between BAT and skeletal muscle ^{18}F-FDG activity related parameters and ad-libitum energy intake (Figure 31), even after adjustment of the analyses by the date when the PET-CT was performed, sex, lean mass and fat mass (Table 20). In addition, no significant associations were found when studying the association of BAT and skeletal muscle related parameters and energy intake estimated with the dietary recalls (Figure 32). These results remained even after adjustment for the date when the PET-CT was performed, sex, lean mass and fat mass (data not shown), or when excluding non-plausible reporters (17 under-reporters, data not shown).

Figure 33 shows the association of body composition parameters with the ad-libitum energy intake. The ad-libitum energy intake was positively associated with lean mass and lean mass index (both $P<0.001$), and negatively related to fat mass percentage ($P<0.001$). BMI, fat mass and fat mass index were not associated with ad-libitum energy intake (all $P>0.15$). When the association was studied with the energy intake estimated with the dietary recalls, lean mass was positively associated, and fat mass percentage negatively associated (both $P<0.011$, Figure 34). Fat mass index was negatively associated

to the energy intake obtained in the dietary recalls (P=0.010), although this association did not remain when excluding non-plausible reporters from the analyses (data not shown). BMI, fat mass and lean mass index were not associated with the energy intake estimated with the dietary recalls (all P>0.1), although lean mass index was positively associated with energy intake when excluding non-plausible reporters (P=0.015; data not shown).

Table 21 shows the interactions of BAT and skeletal muscle activity related parameters with time on the appetite related sensations. No significant interactions were observed for any of the BAT related parameters and time for hunger and fullness sensations (all P>0.28; Figure 35), including all, deep and cervical muscles activity (all P>0.42; Figure 36). We observed marginal significance for the interaction between cold-sensitive muscles glucose uptake and time for hunger sensations (P=0.06), which was corroborated by an interaction of Group*Time when comparing the highest and the lowest tertiles (P=0.034; Figure 36). Moreover, the AUC for fullness was positively correlated with cold-sensitive muscles glucose uptake (P=0.033, data not shown), and marginally associated with all, deep and cervical muscles activity (all P<0.067, data not shown).

Finally, we observed a significant association between the energy intake in the ad-libitum meal and the energy intake estimated with the dietary recalls (P=0.001; Figure 37), which remained after excluding non-plausible reporters.

Table 20. Associations of ad libitum energy intake (kcal) with brown adipose tissue (BAT) and skeletal muscle cold-induced ^{18}F Fluorodeoxyglucose uptake.

	Model 1			Model 2			Model 3		
	β	R^2	P	β	R^2	P	β	R^2	P
BAT volume (ml)	1.02	0.03	0.13	0.40	0.26	0.50	0.49	0.28	0.43
BAT metabolic activity	0.09	0.01	0.42	0.07	0.26	0.50	0.09	0.28	0.41
BAT SUVmean (g/ml)	-15.29	0.01	0.47	3.76	0.26	0.84	9.67	0.28	0.61
BAT SUVpeak (g/ml)	-1.76	0.01	0.71	0.50	0.26	0.91	1.73	0.28	0.69
All muscles SUVpeak (g/ml)	-6.56	0.01	0.97	19.39	0.26	0.91	-41.47	0.28	0.82
Deep muscles SUVpeak (g/ml)	26.27	0.01	0.85	36.08	0.26	0.76	12.80	0.28	0.91
Cervical muscles SUVpeak (g/ml)	0.71	0.01	1.00	46.26	0.26	0.68	25.90	0.28	0.82
Cold sensitive muscles SUVpeak (g/ml)	2.02	0.01	0.99	72.13	0.26	0.54	53.48	0.28	0.66

Unstandardized β, R^2, and P from multiple linear regressions. Model 1: Adjusted by date when BAT assessment was performed. Model 2: Model 1 plus sex. Model 3: Model 2 plus fat mass and lean mass. SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

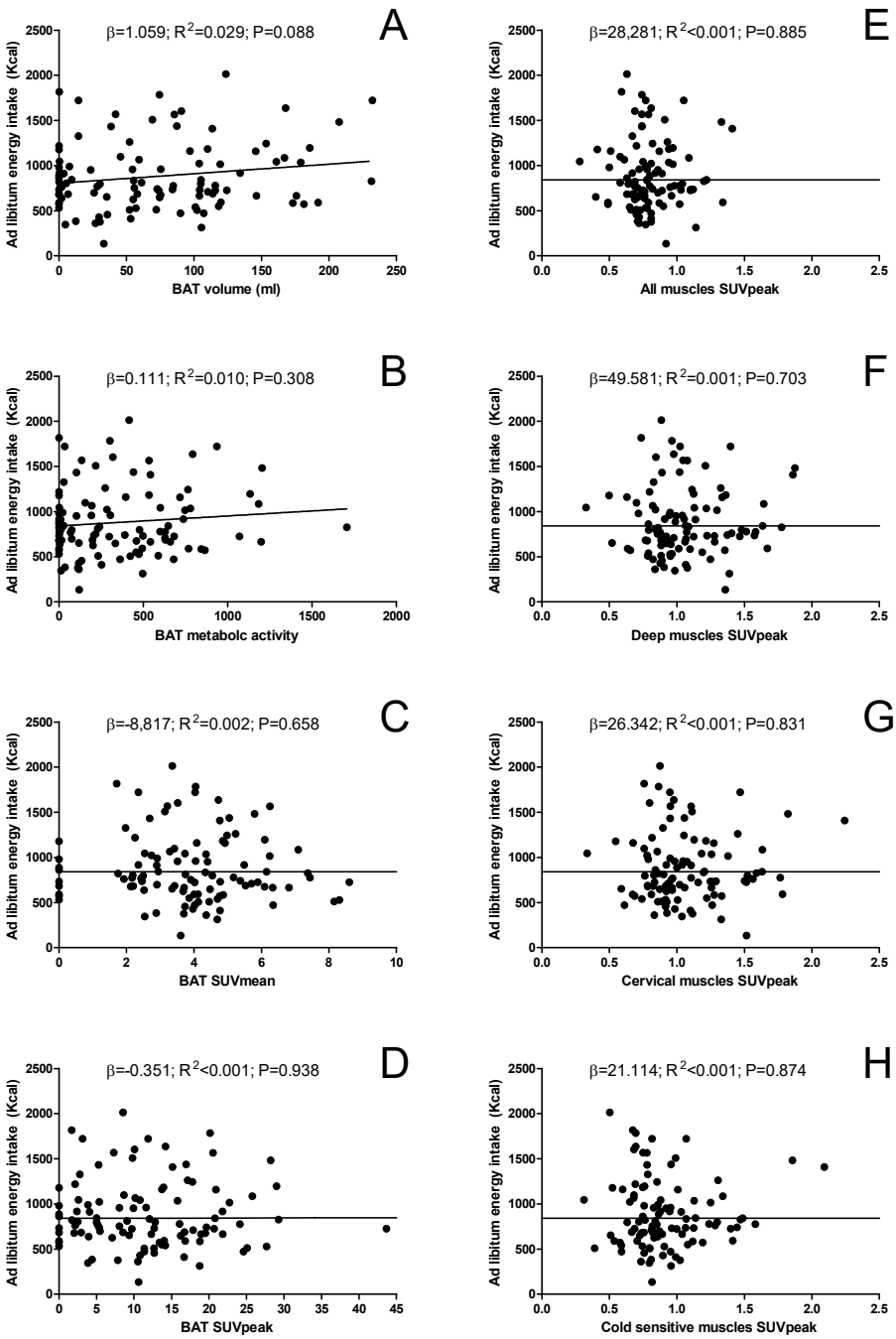

Figure 31. Associations of ad libitum energy intake (kcal) with brown adipose tissue (BAT) and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation. Unstandardized β, R2, and P from simple linear regressions. SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

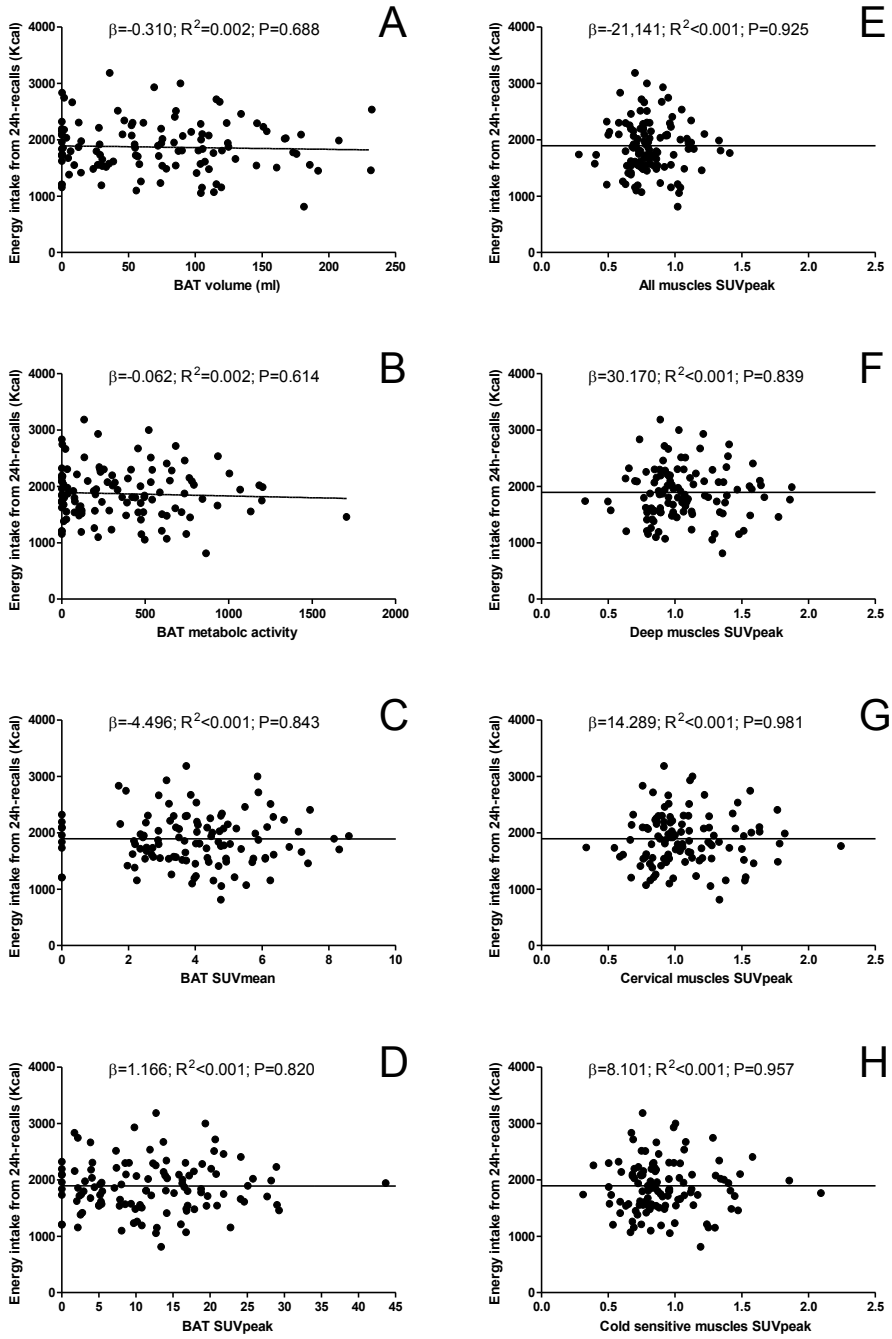

Figure 32. Associations of energy intake (kcal) from the 24 hours dietary recalls (24h-recalls) with brown adipose tissue (BAT) and skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a personalized cold stimulation. Unstandardized β, R2, and P from simple linear regressions. SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

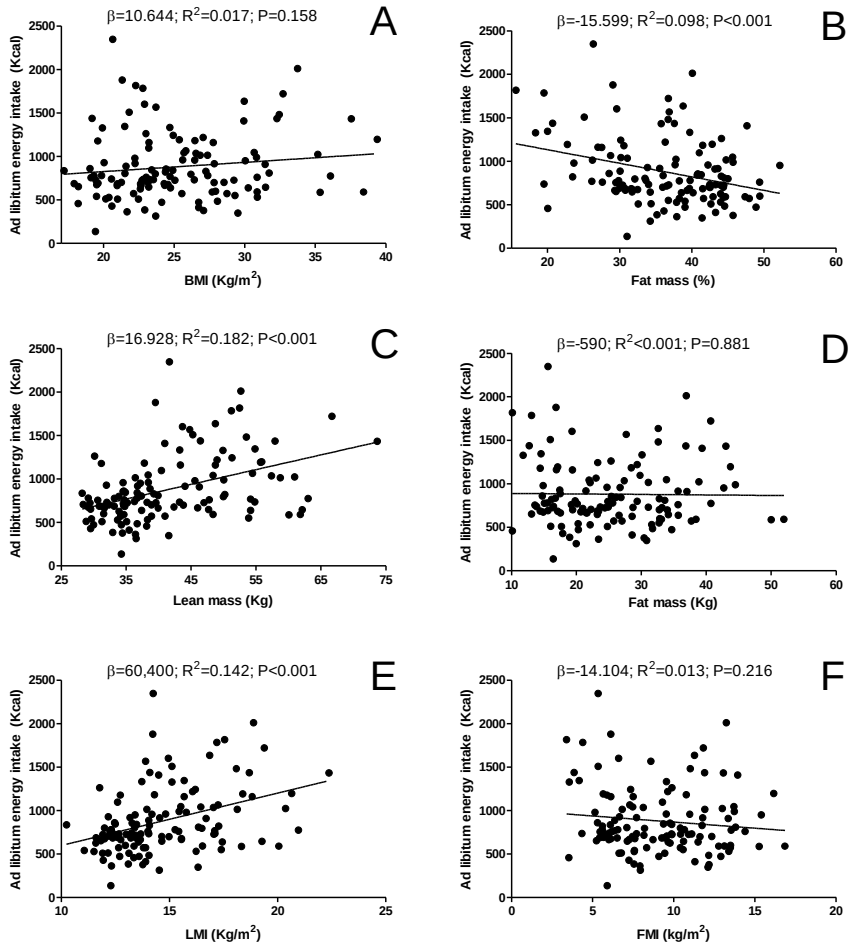

Figure 33. Associations of ad libitum energy intake (kcal) with body composition. Unstandardized β, R2, and P from simple linear regressions. BMI: Body mass index; LMI: Lean mass index; FMI: Fat mass index; VAT: Visceral adipose tissue.

Table 21. Interaction effect of brown adipose tissue (BAT) and skeletal muscle cold-induced ^{18}F-Fluorodeoxyglucose uptake with hunger and fulness sensations.

	Hunger P for interaction	Fullness P for interaction
BAT volume (ml)	0.88	0.30
BAT metabolic activity	0.85	0.45
BAT SUVmean (g/ml)	0.54	0.28
BAT SUVpeak (g/ml)	0.49	0.54
All muscles SUVpeak (g/ml)	0.44	0.91
Deep muscles SUVpeak (g/ml)	0.63	0.86
Cervical muscles SUVpeak (g/ml)	0.42	0.84
Cold sensitive muscles SUVpeak (g/ml)	0.06	0.78

P from a two-factor mixed ANOVA (Time x BAT/Muscle ^{18}F-FDG activity). SUV: Standardized uptake value; All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major.

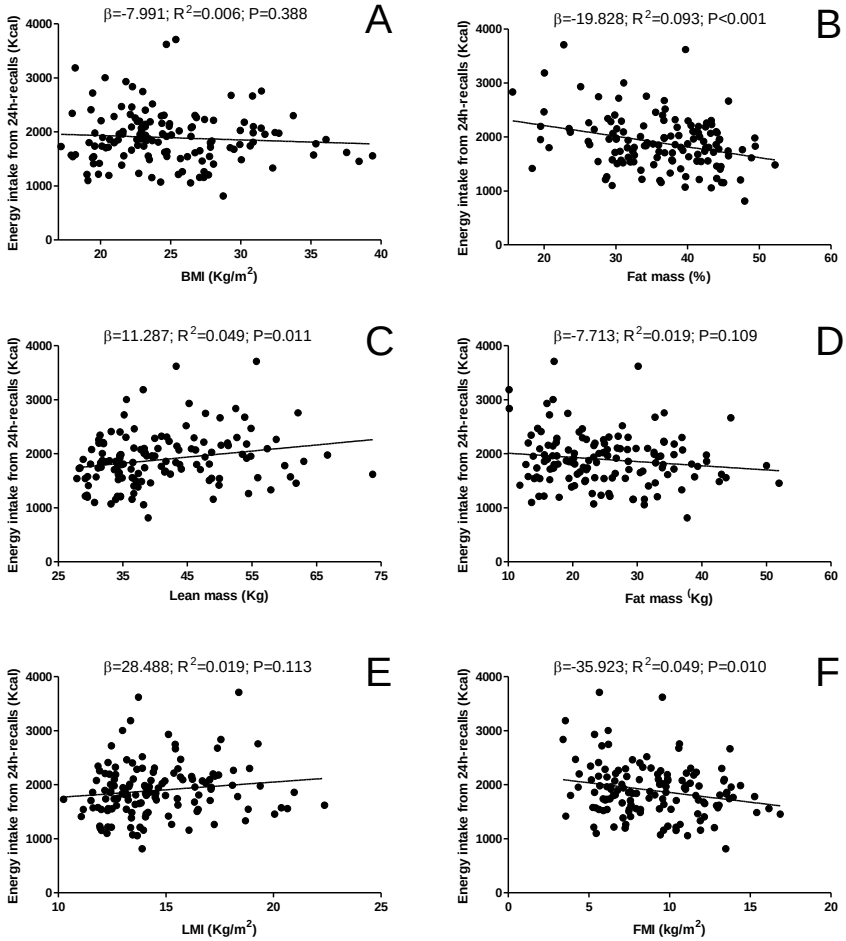

Figure 34. Associations of energy intake (kcal) from the 24 hours dietary recalls (24h-recalls) with body composition. Unstandardized β, R2, and P from simple linear regressions. BMI: Body mass index; LMI: Lean mass index; FMI: Fat mass index; VAT: Visceral adipose tissue.

DISCUSSION

We show, for the first time, that BAT volume and ^{18}F-FDG activity after a personalized cold stimulation are not associated with energy intake, both assessed by an ad-libitum meal or 24-h dietary recalls. Of note, both methods for assessing energy intake were sensitive enough to detect an already well established positive association between lean mass and energy intake [18]. This, together with the lack of interaction between BAT related variables and time on subjective appetite sensations, suggest that BAT is not significantly involved in human appetite regulation, which is consistent with studies showing a negligible impact of BAT on human EE [19–22]. Similarly, skeletal muscle glucose uptake was not associated with energy intake. However, our data suggest that muscle NST could be related to the appetite subjective sensations after a meal, although more studies are needed to fully elucidate this hypothesis.

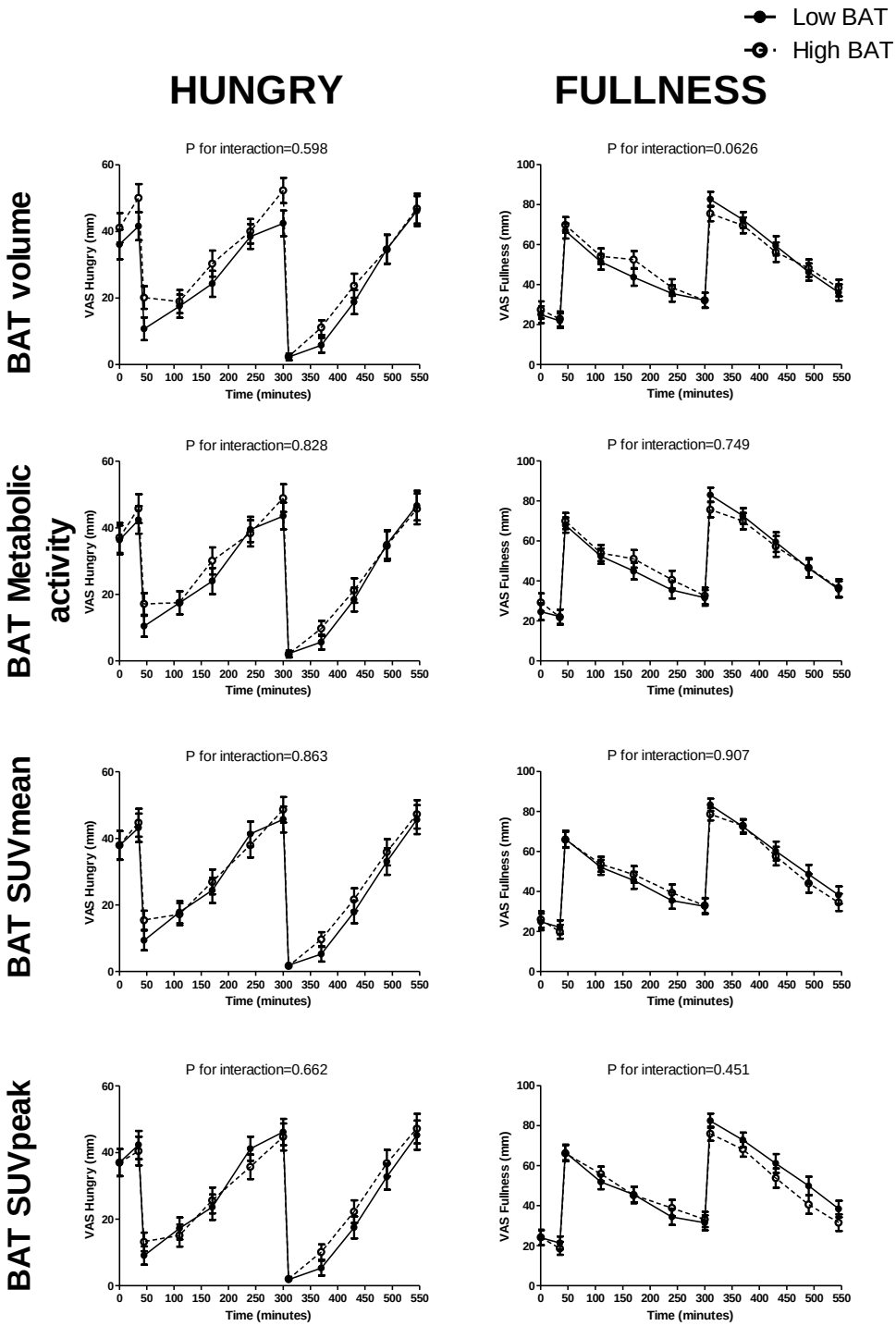

Figure 35. Kinetics of hunger and fullness sensations by brown adipose tissue (BAT) related parameters. High and low muscle activity groups are the higher and lower tertiles of ^{18}F-FDG activity. P from two-factor mixed ANOVA (Time x Group). VAS: Visual analogue scale.

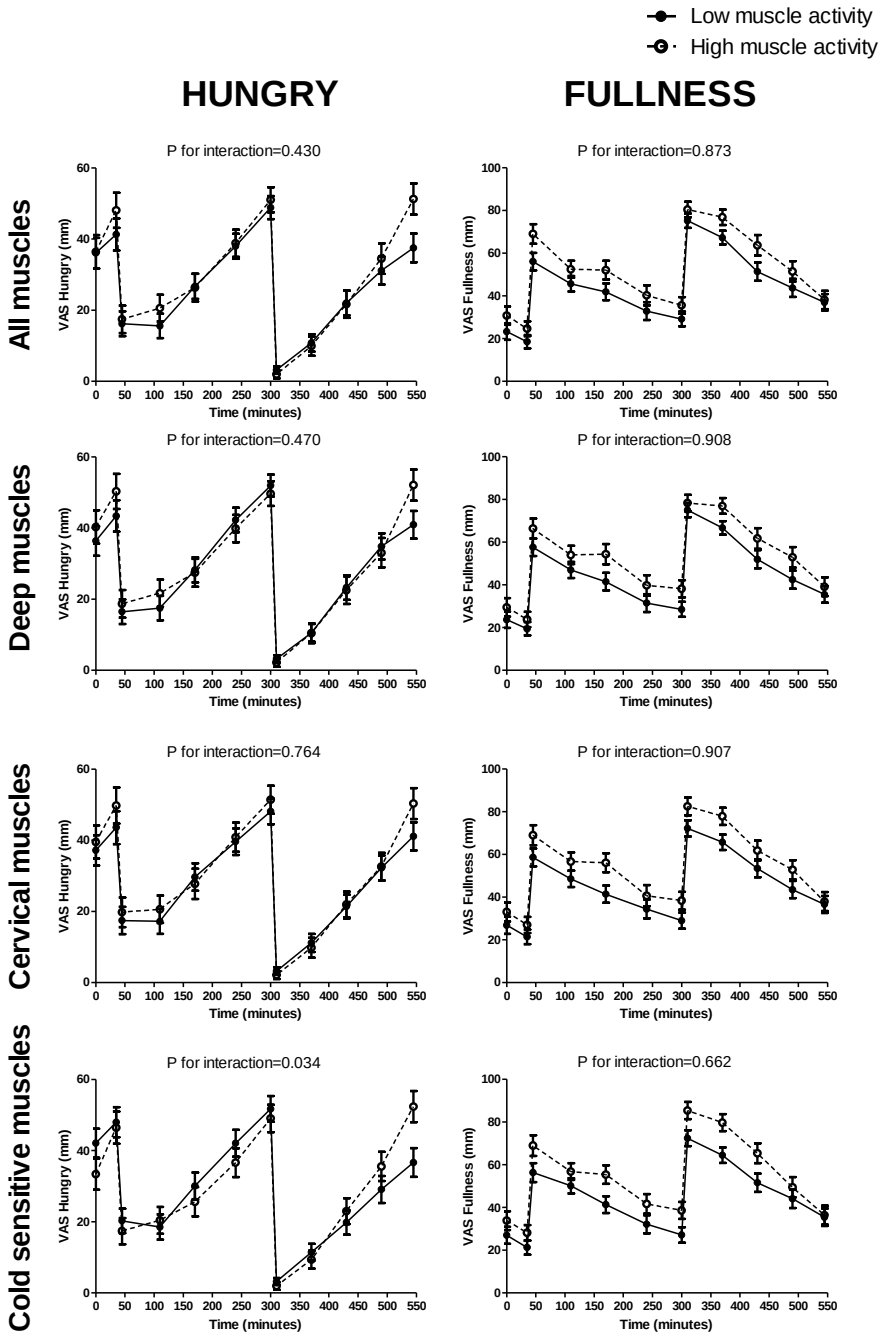

Figure 36. Kinetics of hunger and fullness sensations by cold-induced skeletal muscle activity. High and low muscle activity groups are the higher and lower tertiles of ^{18}F-FDG activity. P from two-factor mixed ANOVA (Time x Group). All muscles: paracervical, sternocleidomastoid, scalene, longus colli, trapezius, parathoracic, supraspinatus, subscapular, deltoid, pectoralis major, and triceps brachii; Deep muscles: paracervical, scalene, longus colli, paravertebral, subscapular; Cervical muscles: paracervical, sternocleidomastoid, scalene, longus colli; Cold sensitive muscles: sternocleidomastoid, scalene, longus colli, pectoralis major. VAS: Visual analogue scale.

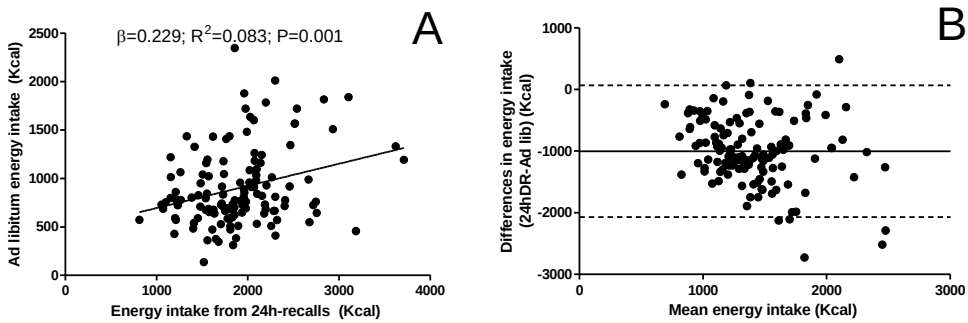

Figure 37. Congruent validity between the lunch ad libitum energy intake and the energy intake from the 24 hours dietary recalls. Panel A: Unstandardized β, R2, and P from simple linear regressions; Panel B: Bland-and-Altman plot.

It is already known that body composition is related to energy intake, with fat free mass, but not fat mass, predicting energy intake [18]. Indeed, BMR seems to mediate the association between lean mass and energy intake [9]. Therefore, BAT could be considered as a third compartment of this body composition model, since it shares histological characteristics with WAT [23], but shares ontogenic origin and thermogenic role with muscle [24]. We did observe the already well proven relationship between lean mass and ad libitum energy intake [18]. However, we failed to observe any association between any BAT-related parameter and energy intake, both assessed by an ad-libitum meal or 24 hours dietary recalls.

This lack of association is consistent with previous studies showing that BAT EE elicited by mild cold or meal ingestion can only account for 10-15 kcal/day if continuously and maximally activated [19–21]. Paradoxically, in these studies, BAT presents higher EE, blood flow up-regulation and fractional substrates uptake per unit of volume that any other measured tissue [7,20,21]. However, the relatively small volume of BAT thought to be present in human adults [25] explain such a small portion of whole-body EE (accounting for less than 1% of total EE) [20,21]. However, it cannot be discounted that human BAT exhibits endocrine connection with the appetite regulation system [13], as it occurs in mice [14,15]. Conversely, the scarce BAT volume in the adult human probably explains why BAT volume or activity were not associated with energy intake or appetite related sensations. Nonetheless, it should be noted that new technologies for BAT in vivo quantification are needed to further confirm the small volume of human BAT shown in previous studies [2].

In addition, it should be noted that we measured energy intake and appetite related sensations in a warm environment, and thus, we cannot know whether BAT would be associated with energy intake or appetite sensations in cold environments. Indeed, an earlier study suggested that EE increases induced by cold might not be compensated by energy intake or appetite related sensations in the short-term [26]. In contrast, cold acclimation in mice increases both EE and intake to a similar extent [11], suggesting that cold exposure increases energy intake thereby compensating for the increase EE, although maybe not in the very short-term. Whether this applies to humans remains to be elucidated. Moreover, it should be noted that BAT activity and EE are quantitatively

similar when induced by mild cold (like the one used for BAT assessment in our study) and a mixed meal [19]. Therefore, it seems plausible to assume that BAT ^{18}F-FDG activity after an individualized cold stimulation is a valid marker of BAT post-prandial activity. Indeed, as stated in the current position statement for human BAT assessment [27], an individualized cold exposure before a ^{18}F-FDG PET-CT is still considered the gold-standard for BAT volume determination [2,27].

Muscle thermogenesis is currently thought to be the main contributor to CIT, even in mild exposures in which shivering is minimized [20,22]. Furthermore, an anatomical dimorphism has been reported by several studies, showing that some muscles groups are specially active in response to cold [7,20,22]. Whether this augmented metabolism is mediated by shivering [22] or non-shivering [28] mechanisms is still unknown. Additionally, it has been suggested, that this muscle metabolism could be partly mediated by endocrine signals released from BAT [20,29]. Interestingly, we found a trend towards an interaction between the skeletal muscle ^{18}F-FDG activity after an individualized cold stimulation in those highly metabolic muscles groups and the time course of changes in hunger sensation, and an association between the AUC for fullness sensations and skeletal muscle ^{18}F-FDG activity after an individualized cold stimulation. This raises the possibility that skeletal muscle, rather than BAT, could be the tissue responsible for both EE and energy intake stimulation in response to cold. Likewise, it could be that cold exposure would be a useful physiological state for studying mechanistic connections explaining the link between fat free mass and energy intake [18].

Our results should be considered with a degree of caution since some limitations are present. We studied young healthy adults, hence we do not know whether these findings extend to older or unhealthy individuals. Moreover, we are aware that energy intake estimations by dietary recalls might present important bias and imprecision, and that a single ad libitum meal is less accurate for estimating daily energy intake than a 24 h ad libitum procedure. However, both the ad-libitum meal and the dietary recalls were sensitive enough to detect the already known relationship between lean mass and energy intake and were associated to each other (Figure S3). Moreover, both the ad libitum meal and visual analogue scales have been proven to be reliable and valid methods for assessing appetite regulation [30,31].

In summary, for the first time, we found that BAT volume and ^{18}F-FDG activity are not associated with energy intake or meal-induced appetite related sensations in young healthy adults. These results together with previous studies, suggest that BAT does not play an important role in energy balance regulation in humans. On the other hand, our results suggest that muscle thermogenesis could be linked to appetite regulation, as it is to EE, although more studies are needed to confirm this intriguing hypothesis.

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SECTION 2

ROLE OF BROWN ADIPOSE TISSUE IN BODY COMPOSITION

Association of brown adipose tissue with bone mineral density

STUDY 7

BACKGROUND

BAT has been suggested to play a role in bone metabolism, possibly stimulating bone anabolism [1,2]. Of note, obesity is characterized by a poor bone metabolism [3], and therefore, the decreased BAT volume and activity associated with obesity could partially mediate the obesity-induced impairments in bone metabolism. Bredella et al. [4] showed, for the first time, in a group of 15 women including healthy women and women with anorexia nervosa, that BMD was higher in those with detectable BAT after a cold stimulation than in those without. Furthermore, they found a positive association between BAT volume and BMD. Lee et al. [5] reported a sexual dimorphism on the association between BAT assessed after a cold exposure and BMD. They showed positive associations between BAT volume and lumbar and whole-body BMD in women but not in men. Other studies have also reported positive associations between BAT volume and the bone cross sectional area in children [6] and adults [7]. However, these associations disappeared when adjusting by muscle mass [6,7].

Several plausible mechanisms could explain the observed the relationship between BAT and BMD in humans [1,2]. Firstly, transcriptional factors such as the forkhead box protein C2 (FOXC2) are involved in both browning of white fat and osteogenic process [8]. Moreover, an endocrine connection between BAT and bone seems to exist [2]. Liu et al. [9] showed that the follicle-stimulating hormone could promote both BAT thermogenesis and bone mass increases, at least in mice. Moreover, irisin seems to induce browning and promote osteogenesis during lineage-specific differentiation *in vitro* [10]. Some bone morphogenic proteins recruit BAT, either by direct or indirect mechanisms [11], and are also able to induce ectopic bone formation [12]. Finally, since BAT activity improves overall energy homeostasis (e.g. improves glucose metabolism), which in turn could lead to a pro-osteogenic environment, we cannot exclude the possibility of BAT affecting bone structure by an indirect mechanism [2].

Currently, the gold standard for quantifying human BAT volume and activity is ^{18}F-FDG PET-CT preceded by cold stimulation [13]. However different methodological approaches have been used to perform and analyze ^{18}F-FDG PET-CT scans [14]. Of note, these methodological differences can have a profound impact on BAT volume and ^{18}F-FDG activity estimations [15]. In 2016, a panel of experts launched a set of recommendations on how to perform and analyze ^{18}F-FDG PET-CT for BAT assessment (i.e. BARCIST 1.0) [13]. Crucially, these recommendations include some considerations that were not followed by most of the previously published studies [13]. Therefore, previous assumptions and findings, such as the BAT and BMD relationship, should be re-addressed.

We aimed to study the association of BAT volume and activity, assessed following the current recommendations [13], with lumbar and whole-body BMD in young healthy adults.

METHODS OVERVIEW

General overview

A total of 98 (68 women) healthy adults aged 18-25 years old participated in the present study (table 22). The participants were enrolled in the ACTIBATE study [16]. Table 23 shows the methodology overview. For a more detailed methods description see "Methods" section.

Table 22. Descriptive characteristics of participants.

	All (n=98)		Men (n=30)		Women (n=68)	
Age (years)	22.0	(2.2)	22.5	(2.2)	21.9	(2.1)
BMI (kg/m^2)	24.3	(4.5)	26.7	(5.3)	23.2	(3.5)
Lean mass (Kg)	40.3	(9.3)	51.6	(7.1)	35.3	(4.5)
Fat mass (Kg)	24.0	(8.5)	24.8	(10.5)	23.6	(7.4)
Fat mass (%)	35.6	(7.3)	30.2	(7.1)	37.9	(6.0)
Total body BMD (g/cm^2)	1.12	(0.10)	1.20	(0.09)	1.08	(0.08)
Lumbar spine BMD (g/cm^2)	1.06	(0.11)	1.03	(0.12)	1.07	(0.11)
BAT volume (cm^3)	71.0	(57.1)	78.1	(64.6)	67.8	(53.6)
BAT SUV mean	3.93	(1.92)	3.26	(1.22)	4.22	(2.10)
BAT SUV peak	11.98	(8.38)	9.99	(6.84)	12.86	(8.89)
Physical activity (counts/5seconds)	28.9	(5.4)	29.9	(5.9)	28.3	(5.2)

Data are presented as means (standard deviation). BMI: Body mass index; BMD: Bone mineral density; BAT: Brown adipose tissue; SUV: Standardized uptake value.

Table 23. Study 7 methodology.

GENERAL INFORMATION	
General aim	To study the association of BAT with bone mineral density
Design	Cross-sectional
Cohort and participants	ACTIBATE (n=98)
OUTCOMES INCLUDED IN THE STUDY	
Outcome	Variables included
PET-CT	BAT volume (-190/-10 HU; Ind. SUV threshold.) BAT SUVmean (-190/-10 HU; Ind. SUV threshold.) BAT SUVpeak (-190/-10 HU; Ind. SUV threshold.) BAT volume (-190/-10 HU; SUV threshold=2) BAT SUVmean (-190/-10 HU; SUV threshold=2) BAT SUVpeak (-190/-10 HU; SUV threshold=2)
BCa	Total body BMD Total body less head BMD Lumbar spine BMD Height Fat mass percentage BMI
ACC	Physical activity
Texpo	Personal environmental temperature

BAT: Brown adipose tissue; PET-CT: Positron emission tomography-Computerized tomography; Ind: Individualized; SUV: Standardized uptake value; BC: Body composition assessment; BMD: Bone mineral density; BMI: Body mass index.

Statistical analyses

The distribution of the variables was verified using the Shapiro–Wilk test, skewness and kurtosis values, visual check of histograms, Q-Q, and box plots. The descriptive data are reported as mean and SD. The differences between men and women were tested with the Student *t*-test. We used simple linear regression models (Model 1) to test the association between BAT -related parameters and BMD (total body, lumbar spine, and total body less head). We also used multiple linear regression models to test these associations adjusting by height (Model 2), height and fat mass percentage (Model 3), and height, fat mass percentage, and PA (Model 4). Further analyses using lean mass, BMI, personal environmental temperature, evaluation date and frequency of alcohol consumption (self-reported) were tested. 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.

RESULTS

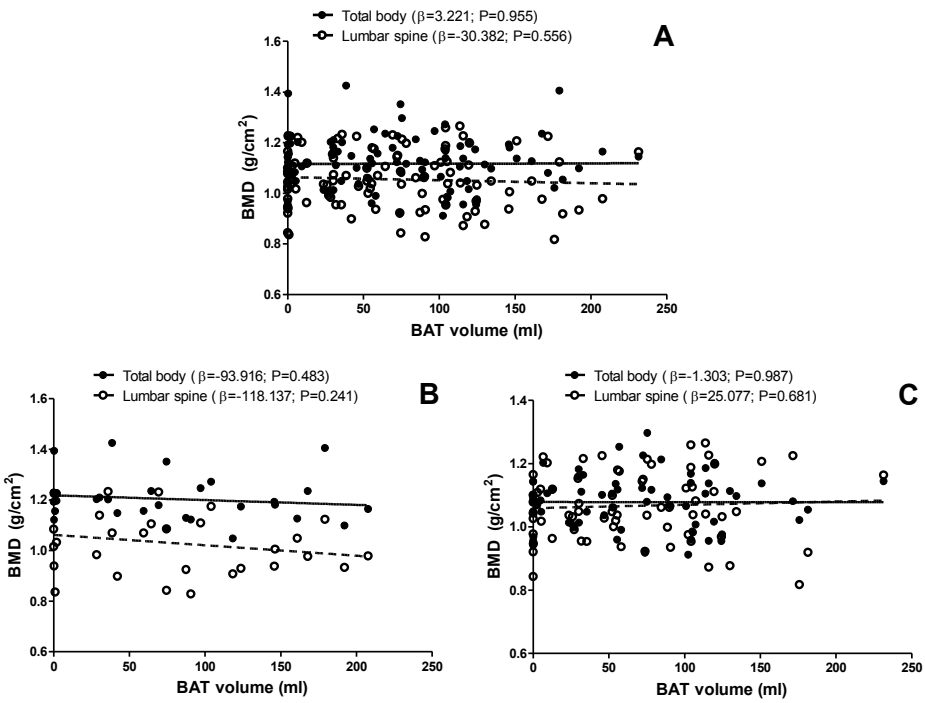

Figure 38. Association between brown adipose tissue (BAT) volume and bone mineral density (BMD). Panel A: all participants; Panel B: male; Panel C: female. β and P from a simple linear regression analysis.

Figure 38 shows the association between BAT volume and BMD (total body and lumbar spine). No significant associations were found for the whole sample (P>0.5; Figure 38A) in neither men (P>0.2; Figure 38B) nor women (P>0.5; Figure 38C). Similar results were

found when studying the association between BAT volume and total body less head BMD (data not shown).

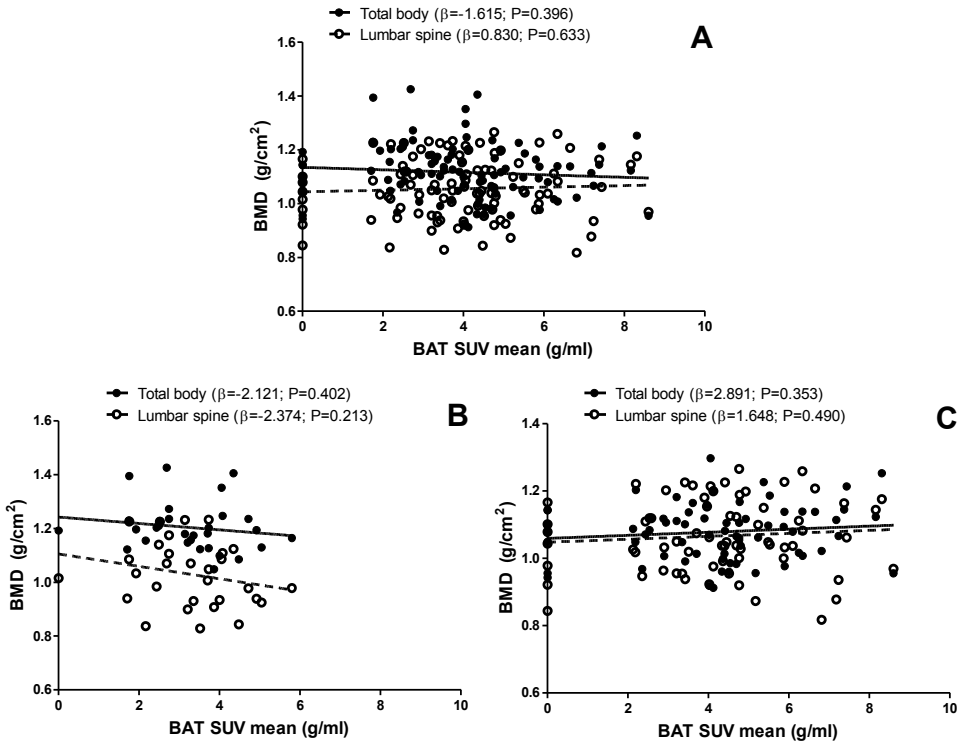

Figure 39. Association between brown adipose tissue (BAT) mean 18F-FDG activity and bone mineral density (BMD). Panel A: all participants; Panel B: male; Panel C: female. β and P from a simple linear regression analysis. SUV: Standardized uptake value.

The associations between BAT mean (SUV mean) and maximum (SUV peak) activity and BMD (total body and lumbar spine) are shown in Figures 39 and 40. No significant associations were found for the whole sample (all P>0.1; Figures 39A and 40A) in neither men (all P>0.2; Figures 39B and 40B) nor women (all P>0.4; Figures 39C and 40D). Also, no significant associations were observed when studying the association between BAT ^{18}F-FDG activity and total body less head BMD (data not shown). Similar results were found when using SUV_{LBM} instead of SUV_{BM} for both BAT mean and maximal ^{18}F-FDG activity (data not shown).

The regression models for studying the association of BAT-related parameters with total body and lumbar spine BMD and are shown in Tables 24 and 25, respectively. All associations between BAT-related parameters and total body and lumbar spine BMD remained non-significant after adding height (Model 2, all P>0.2), fat mass percentage (Model 3, all P>0.3), BMI (Model 4, all P>0.25) and PA (Model 5 all P>0.09) as covariates.

All the analyses were repeated using the fixed SUV threshold (i.e. SUV≥2) for BAT quantification [5] instead of the personalized SUV threshold [13], and the results persisted (data not shown). We also tested whether Model 3 yielded different results introducing lean mass as covariate instead of fat mass percentage, and similar results were found

(data not shown). We further adjusted the regressions by the personal environmental temperature, yet the results persisted (data not shown). In addition, Model 5 remained non-significant when introducing the evaluation date, or the frequency of alcohol consumption (self-reported), instead of the PA as covariates.

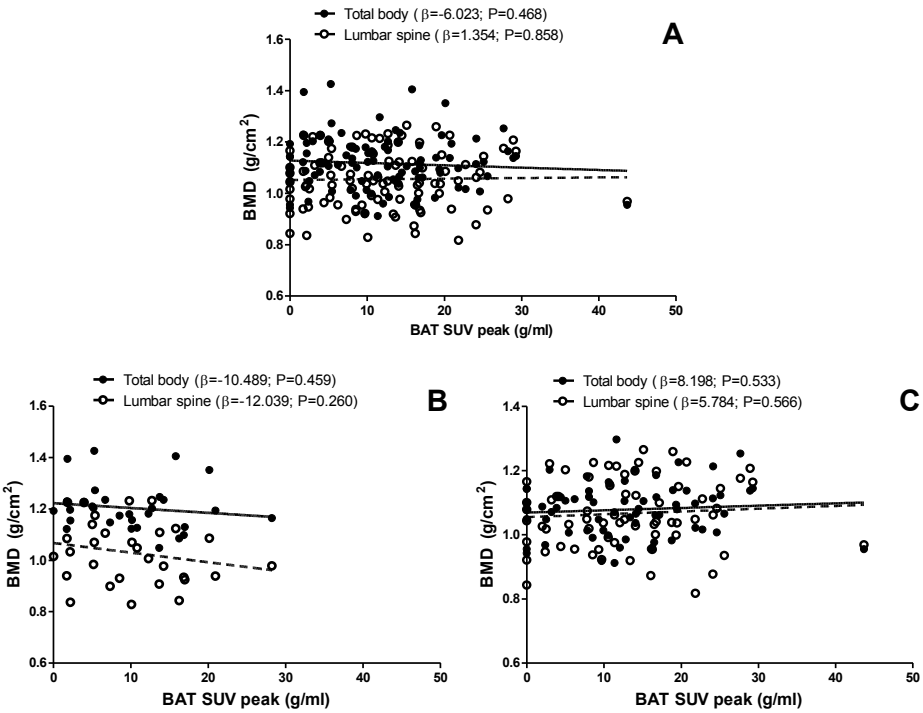

Figure 40. Association between brown adipose tissue (BAT) maximal ^{18}F-FDG activity and bone mineral density (BMD). Panel A: all participants; Panel B: male; Panel C: female. β and P from a simple linear regression analysis. SUV: Standardized uptake value.

DISCUSSION

The present study shows that BAT volume and ^{18}F-FDG activity after a personalized cold exposure are not associated with total body and lumbar spine BMD in young healthy adults. We also showed that this lack of significant relation is present in both men and women, and that it is independent of height, body composition, and PA. These findings disagree with previous studies which showed a positive association between BAT volume and BMD in women [4,5]. Of note, we assessed BAT volume and ^{18}F-FDG activity following current recommendations [13], and we conducted sensitivity analyses. The assumption of a positive relation between BAT and BMD in adults might be biased by methodological issues and/or be only applicable to some pathological conditions (e.g. anorexia nervosa), while it seems not to exist in young healthy adults.

Lee et al. [5] reported no association between whole-body and lumbar spine BMD and BAT volume in a group of 14 men, which concurs with our findings in men. However, both Lee et al. [5] and Bredella et al. [4] showed a positive association between BAT volume and BMD in two groups of 10 and 15 young women respectively. Besides the relatively small

sample size, which could have biased their results, methodological differences between our study and the ones by Lee et al. and Bredella et al. could explain such a discrepancy [13]. On the other hand, it should be noted that Bredella et al. [4] included 5 healthy women and 10 women suffering or recovered from anorexia nervosa. Of note, 4 out of 5 (80%) healthy women presented detectable BAT after the cold stimulation, whereas only 2 out of 10 (20%) women suffering or having recovered from anorexia nervosa presented detectable BAT after the cold stimulation [4]. Thus, it is biologically plausible that metabolic changes occurring in anorexia nervosa, affecting bone metabolism [17], would be driving the BAT - BMD association, which would not apply to healthy people (like the one included in our study).

Table 24. Association of brown adipose tissue (BAT) volume and 18F-FDG activity with total body bone mineral density.

	All			Men			Women		
	β	R^2	P	β	R^2	P	β	R^2	P
BAT vol									
Model 1	3.221	0.000	0.955	-93.916	0.018	0.483	-1.303	0.000	0.987
Model 2	-16.005	0.005	0.798	86.036	0.029	0.528	-8.841	0.007	0.913
Model 3	39.745	0.063	0.544	-35.495	0.227	0.777	21.425	0.044	0.794
Model 4	-36.558	0.053	0.557	120.014	0.136	0.367	-6.188	0.023	0.939
Model 5	-233.313	0.383	0.072	271.226	0.576	0.171	146.932	0.441	0.496
BAT SUV mean									
Model 1	-1.615	0.008	0.396	-2.121	0.025	0.402	2.891	0.013	0.353
Model 2	-0.887	0.014	0.673	-2.121	0.025	0.413	2.771	0.014	0.380
Model 3	0.473	0.044	0.832	-1.652	0.073	0.526	3.336	0.023	0.307
Model 4	-0.659	0.020	0.757	-2.286	0.032	0.391	2.727	0.017	0.391
Model 5	-0.501	0.312	0.919	-3.011	0.287	0.540	10.190	0.317	0.271
BAT SUV peak									
Model 1	-6.023	0.006	0.468	-10.489	0.020	0.459	8.198	0.006	0.533
Model 2	-5.186	0.006	0.573	-9.844	0.027	0.496	6.867	0.014	0.606
Model 3	-0.111	0.028	0.991	-8.369	0.042	0.571	9.671	0.026	0.482
Model 4	-4.660	0.007	0.691	-10.289	0.028	0.489	6.815	0.014	0.612
Model 5	-7.978	0.268	0.690	-15.677	0.308	0.397	41.994	0.406	0.231

SUV, standardized uptake value. β unstandardized regression coefficient. Model 1 shows the coefficient from a single linear regression (raw). The analyses were controlled for: height (Model 2); height and fat mass percentage (Model 3); height and body mass index (Model 4); height, fat mass percentage, and physical activity (Model 5).

BAT is radiologically defined as all voxel in an ^{18}F-FDG PET-CT meeting a range of radio-density (i.e. HU) and a threshold of glucose uptake (SUV threshold, e.g. SUV≥2) [13]. Nowadays, there is a consensus on the need to individually adjust the SUV threshold considering body composition [13]. In fasting state, white adipose tissue ^{18}F-FDG uptake is rather low, and thus body fat percentage determines the average amount of ^{18}F-FDG which is available for the rest of tissues (lean tissues). As a consequence, higher SUV values are found in lean tissues of fatter individuals [13]. Lee et al. [5] assessed BAT using a fixed SUV threshold (SUV≥2), which in turn could overestimate BAT volume in the fatter individuals. Since body composition can have a profound impact on BMD [18], using a fixed SUV threshold could have biased the BAT-BMD relation [5]. In order to test this hypothesis, we studied the association between BAT and BMD using both an individualized [13] and a fixed [5] SUV threshold (i.e. 2 g/ml), and we found no significant association with neither SUV threshold.

Another key factor to properly assess human BAT is exposing the participants to a cooling protocol before performing the PET-CT scan [13]. Of note, this cooling protocol

should also be personalized [13,19]. Since cold tolerance is highly variable between individuals, exposing every participant to the same temperature (e.g. 19ºC [5]) before performing the PET-CT could implicate a higher stimulation in those participants with a lower cold tolerance [19]. In order to avoid such a bias, it was proposed to individualize the cooling stimulus to each participant's shivering threshold [13,19]. Shivering threshold should be lower in obese individuals due to a higher insulation capacity [19]. Therefore, exposing every participant to the same temperature could lead to BAT underestimation in fatter individuals [19]. Crucially, since body composition influences both cold tolerance [19] and BMD [3,18], using a personalized cold exposure is mandatory to study the association between BAT and BMD. Of note, neither Bredella et al. [4] nor Lee et al. [5] applied a personalized cold exposure.

Table 25. Association of brown adipose tissue (BAT) volume and 18F-FDG activity with lumbar spine bone mineral density.

	All			Men			Women		
	β	R^2	P	β	R^2	P	β	R^2	P
BAT volume									
Model 1	-30.382	0.004	0.556	-118.137	0.049	0.241	25.077	0.003	0.681
Model 2	-32.780	0.009	0.527	-120.255	0.065	0.238	14.214	0.008	0.824
Model 3	-37.921	0.065	0.454	-61.881	0.237	0.520	10.099	0.043	0.873
Model 4	-27.032	0.053	0.596	-100.083	0.142	0.317	6.582	0.023	0.919
Model 5	-112.278	0.339	0.202	-146.258	0.454	0.487	-92.927	0.454	0.379
BAT SUV mean									
Model 1	0.830	0.002	0.633	-2.374	0.055	0.213	1.648	0.007	0.490
Model 2	0.957	0.016	0.583	-2.382	0.055	0.220	1.496	0.008	0.551
Model 3	0.828	0.046	0.631	-1.884	0.090	0.346	1.445	0.012	0.568
Model 4	0.887	0.021	0.612	-2.342	0.056	0.241	1.658	0.012	0.515
Model 5	4.054	0.357	0.210	-0.418	0.238	0.933	6.244	0.355	0.165
BAT SUV peak									
Model 1	1.354	0.000	0.858	-12.039	0.045	0.260	5.784	0.005	0.566
Model 2	1.606	0.003	0.833	-12.221	0.056	0.259	3.711	0.012	0.726
Model 3	1.099	0.028	0.885	-10.881	0.064	0.337	3.395	0.020	0.749
Model 4	1.423	0.005	0.853	-12.283	0.056	0.270	3.937	0.012	0.714
Model 5	16.890	0.313	0.198	-3.455	0.217	0.856	27.359	0.460	0.105

SUV, standardized uptake value. β unstandardized regression coefficient. Model 1 shows the coefficient from a single linear regression (raw). The analyses were controlled for: height (Model 2); height and fat mass percentage (Model 3); height and body mass index (Model 4); height, fat mass percentage, and physical activity (Model 5).

Finally, besides the health status and the methodological issues for BAT assessment stated above, when interpreting the discrepancies between our study and those of Bredella et al. [4] and Lee et al. [5] differences in the study population should be considered. Participants of our study lived in southern Spain, and therefore, in a hotter climate than those in Bredella et al. [4] (Boston, MA, USA) and Lee et al. [5] (Bethesda, MD, USA) studies. Importantly, it should be note that outdoor temperature profoundly impact BAT detectability [20–23], and therefore could be influencing the lack of association in our study. However, we checked whether the personal environmental temperature of the 7 days before the PET-CT affected the results and failed to observe significant associations. Moreover, it should be noted that participants in Lee et al. [5] were 28±7 years old, which is 6 years older in average than participants in our study. Of note, BAT volume and ^{18}F-FDG activity markedly decline with aging [24,25]. Therefore, it could be that differences in

participants age may also partially explain why we did not observe the association found by Lee et al. [5] study.

Importantly, that the lack of association in our study was independent of several potential confounders, such as body composition, daily temperature exposure, and also PA. PA has a positive impact on BMD [26,27], which is mostly explained by mechano-adaptation [28,29]. However, effects of PA on BMD might be also partially related to the exercise induced browning of marrow fat [30–32]. However, we observed that objectively measured PA did not modify the association between BMD and BAT.

Several mechanisms by which BAT could stimulate bone anabolism have been proposed [1,2], yet, most of available mechanistic studies were performed in murine models [1,2,9,10,33,34], and translation from murine to humans becomes especially difficult in BAT physiology [35]. However, there are also mechanisms by which BAT could negatively influence BMD. The transcriptional regulator and tumor suppressor retinoblastoma-associated protein (pRb) can influence a mesenchymal cell through either an adipocyte or osteoblast lineage [36]. Of note, the loss of pRb translates into an increase of BAT mass and less calcified bone [36,37]. In addition, aberrant high levels of fibroblast growth factor 21 (FGF21), which is a BAT-secreted and a BAT-stimulating protein, are associated with poor bone homeostasis in HIV-1-infected patients [38]. Interestingly, marrow adipose tissue (i.e. adipose tissue depots inside the bone marrow [39]), which seems to be inversely associated with BMD [39–41], seems to exhibit an elevated expression of beige fat markers, including UCP1, HOXC9, PRDM16, TBX1, and DIO2 [33], in some bone specific locations.

This study's findings should, however, be taken with caution as some limitations arise. Firstly, our study is observational and therefore no causality can be established. Secondly, our study only included healthy young people, and hence we do not know whether these results extend to older or unhealthy people. Moreover, our results could be affected by the nature of the F-FDG tracer [42], as every BAT study performed with ^{18}F-FDG PET-CT. However, this technique is currently considered the gold standard for BAT assessment and we followed the most updated recommendations in this study. Finally, instead of a region-specific scan we used the lumbar spine from a whole-body DXA scan, which is not as informative. We did not test the DXA scan coefficient of variation, yet previous studies in healthy adults have shown that the DXA between-day coefficient of variation was <0.1 % [43].

In summary, our study shows that BAT and BMD are not associated in young healthy adults. Moreover, our data support the notion that previously showed associations between BAT and BMD, at least in healthy women, could be explained by methodological issues related to BAT assessment and/or sample size limitations. Nonetheless, further studies are needed to confirm whether an association exists in older or unhealthy people, and experimental studies are warranted to determine whether BAT can really influence BMD in human adults.

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Association of brown adipose tissue with whole-body fat and lean mass

STUDY 8

BACKGROUND

Early studies assessing human BAT by ^{18}F-FDG PET-CT showed an inverse association between BAT volume and whole-body adiposity[1–3], which was later confirmed by other groups. However, already in the 70´s, it was shown that providing an hypercaloric cafeteria diet increased whole-body adiposity as well as BAT volume in rats[4]. This classic study, and others[5–9], provided evidence of an obesity-induced hypertrophy/hyperplasia of BAT, at least when studying wild-type rodents that occurred in the context of *increasing* obesity[10]. High fat feeding, in contrast to its effects on classical BAT, appears to reduce the beige fat content of mice[11], providing an alternative paradigm to explain the negative association between human BAT mass and adiposity. Therefore, the negative association between BAT volume and whole-body adiposity shown by first human PET/CT studies are in contrast with the physiological phenomenon of diet-induced adaptive thermogenesis in rodents. Of note is however that most of the human BAT detected by ^{18}F-FDG seems to be more similar to the murine beige adipose tissue rather than classic BAT [12].

Importantly, recent advances in human BAT assessment methodology suggest that the initially observed inverse associations between human BAT and body adiposity could have been, at least in part, biased by methodological issues. Firstly, initial studies on human BAT, and many of the later studies, performed the ^{18}F-FDG-PET/CT in thermoneutral environments. Current recommendations are to perform the PET/CT after an individualized cold-exposure[13]. Using a single general (i.e. non- individualized) cold-exposure before the PET/CT over or under-estimate the BAT volume and activity of individuals depending on their insulation and cold tolerance capacity (e.g. air at 17°C could maximize NST in one participant, despite being a light stimulus for NST in another) [14]. Of note, since obesity is thought to modify an individual's insulation and/or heat production rate[15], not individualizing cold exposure protocols can systematically bias the relation between BAT and whole-body adiposity [14]. Secondly, the standardized uptake value (SUV), the unit to express the ^{18}F-FDG activity, was traditionally expressed relative to body mass (SUV_{BM}). Currently, it is known that SUV should be expressed relative to lean body mass (SUV_{LBM}) to avoid a body composition bias on glucose uptake assessment[16]. Moreover, the SUV threshold, considered as the minimal radiotracer uptake to quantify a voxel as BAT, should also be expressed relative to lean body mass [13,17], which was not done in most humans studies showing an inverse association between BAT and whole-body adiposity. Finally, it should be noted that using the glucose analogue ^{18}F-FDG, the most frequently used radiotracer for BAT assessment, may also bias the relation between BAT and body composition. Insulin resistant states, such as type 2 diabetes mellitus, might impair BAT ^{18}F-FDG uptake without affecting its oxidative metabolism or fatty acid uptake [18]. Therefore, it is plausible that the inverse association between BAT (assessed by ^{18}F-FDG activity) and whole-body adiposity might also be biased in individuals with obesity-induced insulin resistance.

This study aimed to investigate the association between BAT and body composition in young healthy adults. We assessed BAT volume and ^{18}F-FDG activity by strictly following current methodological recommendations for human BAT analysis and quantification[13].

METHODS OVERVIEW

General overview

A total of 114 (76 women) young healthy adults participated in the study (Table 26). The data were part of the baseline assessment of the ACTIBATE study [19].

Table 26. Characteristics of the study participants.

	ALL (114)		MEN (38)		WOMEN (76)	
Age (years)	22.03	(2.15)	22.35	(2.17)	21.86	(2.13)
BMI (kg/m^2)	24.71	(4.69)	27.16	(5.58)	23.48	(3.63)
Under-weight (n, %)	6	(5.3)	1	(2.6)	5	(6.6)
Normal-weight (n, %)	65	(57.0)	17	(44.7)	48	(63.2)
Over-weight (n, %)	28	(24.6)	10	(26.3)	18	(23.7)
Obese-weight (n, %)	15	(13.2)	10	(26.3)	5	(6.6)
Lean mass (Kg)	41.37	(9.62)	52.36	(6.96)	35.88	(4.87)
LMI (kg/m^2)	14.52	(2.44)	17.12	(2.09)	13.22	(1.29)
Fat mass (Kg)	24.91	(9.05)	26.04	(11.36)	24.35	(7.67)
FMI (kg/m^2)	8.81	(3.01)	8.51	(3.68)	8.96	(2.63)
Fat mass (%)	35.82	(7.43)	30.85	(7.72)	38.30	(5.93)
VAT (g)	331.30	(180.54)	432.00	(180.96)	280.94	(158.90)
BAT volume (ml) (ind-SUVt)	69.38	(58.23)	78.12	(67.28)	65.02	(53.07)
BAT volume (ml) (fix-SUVt)	72.57	(63.31)	76.86	(72.99)	70.42	(58.27)
BAT SUVmean (g/ml) (SUV_{LBM})	3.72	(1.90)	3.24	(1.53)	3.97	(2.02)
BAT SUVmean (g/ml) (SUV_{BM})	2.24	(1.08)	2.08	(0.79)	2.33	(1.19)
BAT SUVpeak (g/ml) (SUV_{LBM})	6.70	(4.77)	6.29	(4.56)	6.90	(4.88)
BAT SUVpeak (g/ml) (SUV_{BM})	11.43	(8.40)	9.89	(7.69)	12.19	(8.68)
scWAT upper back SUVpeak (SUV_{LBM})	0.30	(0.15)	0.34	(0.14)	0.28	(0.15)
scWAT upper back SUVpeak (SUV_{BM})	0.51	(0.25)	0.54	(0.21)	0.50	(0.27)
scWAT tricipital SUVpeak (SUV_{LBM})	0.09	(0.05)	0.12	(0.06)	0.08	(0.03)
scWAT tricipital SUVpeak (SUV_{BM})	0.16	(0.07)	0.19	(0.09)	0.14	(0.05)
Descending aorta SUVpeak (g/ml) (SUV_{LBM})	0.92	(0.20)	1.03	(0.20)	0.86	(0.17)
Descending aorta SUVpeak (g/ml) (SUV_{BM})	1.56	(0.34)	1.63	(0.36)	1.52	(0.32)
HOMA-index	1.88	(1.29)	2.14	(1.71)	1.75	(0.99)

Data are means and standard deviation, unless otherwise stated. BAT: Brown adipose tissue; BM: Body mass; BMI: Body mass index; fix-SUVt: Fixed SUV threshold; FMI: Fat mass index; ind-SUVt: Individualized SUV threshold; LBM: Lean body mass; LMI: Lean mass index; scWAT: subcutaneous white adipose tissue; SUV: Standardized uptake value; VAT: Visceral adipose tissue.

Table 27 shows the methodology overview. For a more detailed methods description see “Methods” section.

Systematic review

We carried out a systematic literature search of human studies reporting an association between BAT and any variable of body composition, or differences between lean and overweight-obese individuals in BAT-related parameters. We screened for human studies published since 2007 when Nedegard et al. [20] published their review confirming the presence of BAT in human adults, until July 15th 2018. We searched in MEDLINE database (through PubMed) using the Medical Subject Heading (MeSH) terms. We included studies written in English and having assessed BAT by means of ^{18}F-FDG-PET-CT in humans. No restrictions were considered regarding study design (cross sectional, case-control, cohort

study) or data collection (prospective or retrospective). We excluded non-original articles. 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. We excluded studies comparing a group of BAT positive (BAT+) participants against a BAT negative (BAT-) group when the authors declared that groups were matched by body composition. Two researchers independently checked the eligibility of the obtained articles, and a consensus meeting was established to clarify the discrepancies. The search terms combination was:

(((("BROWN ADIPOSE TISSUE") OR ("BROWN FAT") OR ("Adipose Tissue, Brown"[Mesh])) AND (("MEN") OR ("WOMEN") OR ("HUMANS") OR ("PET-CT") OR ("PET/CT") OR ("18-FDG")) NOT ((" review "[Publication Type]) OR (" review literature as topic "[Mesh]) OR (" rodent ") OR (" mice ") OR (" mouse ") OR (" Rat ") OR (" rats ") OR (" syndrome ") OR (" Tc-99m ") OR (" 99mTc ") OR (" pregnancy ") OR (" fetal ") OR (" hibernoma ") OR (" carcinoma ")))

Table 27. Study 8 methodology.

GENERAL INFORMATION	
General aim	To study the association of BAT with body composition (fat and lean mass)
Design	Cross-sectional
Cohort and participants	ACTIBATE (n=114)
OUTCOMES INCLUDED IN THE STUDY	
Outcome	Variables included
PET-CT	BAT volume (-190/-10 HU; Ind. SUV threshold.) BAT metabolic activity (-190/-10 HU; Ind. SUV threshold.) BAT SUVmean (-190/-10 HU; Ind. SUV threshold.; SUB_{LBM}) BAT SUVpeak (-190/-10 HU; Ind. SUV threshold.; SUB_{LBM}) BAT volume (-190/-10 HU; SUV threshold=2) BAT metabolic activity (-190/-10 HU; SUV threshold=2) BAT SUVmean (-190/-10 HU; SUV threshold=2.; SUB_{BM}) BAT SUVpeak (-190/-10 HU; SUV threshold=2.; SUB_{BM}) BAT mean radiodensity Upper back subcutaneous WAT SUVpeak Tricipital subcutaneous WAT SUVpeak Descending aorta SUVpeak
BCa	BMI Lean mass Fat mass Fat mass percentage Visceral adipose tissue mass BSA
CVD	HOMA

BAT: Brown adipose tissue; PET-CT: Positron emission tomography-Computerized tomography; Ind: Individualized; SUV: Standardized uptake value; BCa: Body composition assessment; BM: Body mass; LBM: Lean body mass; BMI: Body mass index; BSA: Body surface area; Homeostatic model assessment.

Statistical analyses

The distribution of the variables was verified using the Shapiro–Wilk test, skewness and kurtosis values, visual check of histograms, Q-Q, and box plots. Results are presented as mean ± SD, unless otherwise stated. We used simple linear regression models to test the association between BAT-related parameters and body composition parameters. We also used multiple linear regression models to test these associations adjusting by the date when the PET-CT was performed (Model 1), the date when the PET-CT was performed and sex (Model 2), and the date when the PET-CT was performed, sex and HOMA (Model 3), since insulin resistance could be mediating the associations between BAT and body composition [21,22]. The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 21.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set at <0.05.

RESULTS

We included a total of 65 papers in our systematic literature search (tables 28-30). We identified 27 studies assessing BAT without a cold exposure prior to the PET-CT, 28 studies assessing BAT after a non-personalized cold exposure, and 10 studies assessing BAT after an individualized cold-exposure. Moreover, only one study used SUV_{LBM}, and there are large differences in the radiodensity criteria used to identify BAT (Tables 28-30). Among the studies assessing BAT in thermoneutral conditions, 85.5% (n=22) reported negative relation between BAT and whole-body adiposity, while 5 studies failed to observe any relationship (table 28). Among the studies using a fixed (i.e. non-personalized) cold exposure, 32.1% (n=9) of the studies observed negative relations between BAT and whole-body adiposity, while 15 studies (53.5%) did not find any association, and 4 studies (14.2%) reported positive associations (table 29). Finally, 40% (n=4) of studies assessing BAT after a personalized cold exposure found negative association between BAT and whole-body adiposity, while 60% (n=6) did not observe any relationship (table 30).

In our study, BAT volume was positively associated with BMI, fat mass, fat mass percentage, and VAT, when assessed with either an individualized[13] or fixed SUV threshold[1–3] (all P<0.042, Figure 41). All associations were independent of the date when the PET-CT was performed, sex, and HOMA (Table 31). On the other hand, BAT was not associated with lean mass (all P>0.36) (Table 31).

Figure 42 shows the association between BAT metabolic activity (BAT volume x BAT SUVmean) and body composition. We observed positive associations of BAT metabolic activity with fat mass and fat mass percentage, both when applying an individualized and a fixed SUV threshold (all P<0.028). A positive association was also found between BAT metabolic activity when assessed with a fixed threshold and BMI. Moreover, we observed marginal associations of BAT metabolic activity with BMI when using an individualized SUV threshold, and BAT metabolic activity with VAT mass (using either SUV thresholds), yet it did not reach statistical significance. No associations were observed between BAT metabolic activity and lean mass (all P>0.8). Moreover, the association remained in the adjusted models (accounting for the date when the PET-CT was performed, sex, and HOMA).

Table 28. Human studies investigating the association of brown adipose tissue assessed with 18F-Fluordeoxyglucose (^{18}F-FDG) Positron Emission tomography (PET)-computerized tomography (CT) and no previous cold-exposure with body composition.

Study	SUV threshold	HU range	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m^2)	BC meth	Results
No cold exposure: Negative association between BAT and whole-body adiposity								
Shao et al.[54]	> 1 (SUVmax)	-100/-10	2944 (1178)	Men: 54.9 ± 12.4 Women: 54.9 ± 12.3	1858 healthy and 1086 cancer patients.	NR	BMI	BAT+ participants had lower BMI than BAT-. BAT SUVmax was inversely correlated to BMI.
Huang et al. [55]	2,5	-250/-50	1740 (723)	BAT+: 40.6 ± 2.7 BAT- 53.8 ± 0.3	Cancer patients (most of them)	BAT+: 21.5 ± 0.6 BAT-: 24.0 ± 0.1	BMI	BAT+ participants had lower BMI than BAT-. No significant association between the total metabolic activity of BAT (n=30, only considering BAT+), and BMI.
Chalfantet al. [56]	NR	NR	32 (12)	13.8 ± 3.6	Pediatric oncology patients	BMI for age percentile 69.0 ± 29.4	CT	No differences in BMI between BAT+ and BAT- participants. BAT+ participants had a lower percentage increase in subcutaneous fat mass compared with BAT-. BAT+ participants had a lower percentage increase in VAT with respect to the BAT-. BAT+ participants had a lower percentage increase in weight than BAT-. No significant differences in weight, BMI-for age-percentile, SAT and VAT between BAT+ and BAT- at disease-free follow up.
Zhang et al. [57]	2	-250/-50	124 (80)	BAT+ men: 35.5 ± 7.8, BAT- men: 39.9 ± 6.4, BAT+ women: 38.0 ± 6.7, BAT- women: 43.5 ± 7.3.	NR (apparently patients going for diagnosis)	BAT+ men: 21.3 ± 2.5 BAT- men: 24.2 ± 4.3 BAT+ women: 21.3 ± 2.2 BAT-women: 22.5 ± 2.5	BMI and BIA	BAT+ participants had lower BMI than BAT- BAT+ male participants had less fat mass percentage than BAT- males. BAT+ participants had lower WC than BAT-.
Takx et al. [58]	2	-250/-50	443 (248)	55 (44–66)	Patients	26 (23–31)	BMI	BAT volume was inversely correlated to BMI.
Persichetti et al. [59]	2	-250/-50	645 (477)	56.25 ± 15.96	Apparently patients (assisting for cancer detection)	25.23 ± 4.73	BMI	BAT+ participants had lower BMI than BAT-. Positive ^{18}F-FDG BAT was inversely correlated to BMI.

Table 28. (Continued)

Study	SUV threshold	HU range	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m²)	BC meth	Results
Wang et al. [60]	2	-250/-50	4011 (1323)	Men: 47.1 (42.1–54.1) Women: 46.1 (41.1-54.1)	Patients tumor-free	Men: 24.9 ± 3.0 Women: 22.3 ± 3.1	BMI and CT	BAT+ participants had lower BMI than BAT-. Furthermore, BAT was inversely associated with BMI (without adjusting for any confounder). BAT+ participants had lower VAT than BAT-. BAT+ participants had lower total and SAT, and WC than BAT-. Furthermore, BAT was inversely associated with WC (without adjusting for any confounder).
Bredella et al. [61]	70% SUVmax	-250/-50	105 (86)	45.5 ± 16.1	Patients tumor-free	25.3 ± 4.8	BMI	BAT was marginally associated with BMI was (in the group as a whole)
Zhang et al. [62]	2	-250/-50	>500 (NR)	BAT+: 40.00±8.17 BAT-: 44.58±8.06	Patients (who underwent the scans for medical checkup or cancer surveillance)	BAT+: 21.1 ± 2.5 BAT-: 24.8 ± 3.2	BMI	BAT was negatively associated with BMI.
Becker et al. [63]	2,5	-250/-50	1031 (418)	62 ± 14.6	Patients that underwent a PET-CT/scan for diagnostic	24.9 ± 4.8	BMI	BAT was negatively associated with BMI.
Yilmaz et al. [64]	NR	NR	30 (20)	44.2 ± 12.5	Patients with suspected malignancies	23.4 ± 3.9	BMI	BAT SUVmax was inversely correlated to BMI.
Cypess et al. [2]	2	-250/-50	310 (172)	BAT+: 49.8 ± 16.3 BAT-: 59.7 ± 14.8	Patients	BAT+: 25.7 ± 5.2 BAT-: 26.6 ± 5.1	BMI	BAT detection was inversely associated with BMI in univariate analyses, although it was not associated in the multivariate analysis. BMI became an independent predictor of the presence of BAT in the multivariate analysis with increasing age. BMI was not an independent predictor of the presence of brown adipose tissue in the multivariate analysis but became a significant predictor with increasing age.
Au-Yong et al. [65]	NR	NR	3614 (1483)	BAT+: 51.8 (13-88)	Patients (most for cancer staging)	NR	BMI	There was a trend for BAT+ participants to have a lower BMI than BAT- (BMI data available only in 57 participants).

Table 28. (Continued)

Study	SUV threshold	HU range	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m²)	BC meth	Results
Pfannenberg et al. [66]	2	-250/-50	260 (136)	48 ± 1	Patients (diagnostic reasons)	24.5 ± 0.3	BMI	BAT activity was inversely associated with BMI in uni- and multi-variate analyses (when sex and age were also introduced as predictor). However, BMI was not associated with BAT mass (multivariate analyses)
Ouellet et al. [67]	1	-100/-10	4842 (2370)	BAT+: 54.4 ± 0.8 BAT-: 62.7 ± 0.2	Patients with cancer	BAT+: 24.6 ± 0.2 BAT-: 26.8 ± 0.1	BMI	BAT was inversely associated with BMI.
Pace et al. [68]	NR	-250/-50	848 (415)	50.9 ± 16	Patients	BAT+: 23 ± 6 BAT-: 27 ± 6	BMI	BAT+ participants had lower BMI than BAT-.
Lee et al. [69]	2	NR	17 (4)	BAT+: 33 ± 8 BAT-: 67 ± 4	Patients (with head and neck malignancies)	BAT+: 22.2 ± 1.0 BAT-: 25.3 ± 1.1	BMI	UCP-1 mRNA abundance in supraclavicular fat correlated negatively with BMI.
Dinas et al. [70]	NR	NR	40 (14)	52.7 ± 17.5	Patients with cancer	26.4 ± 4.5	BMI	BAT activity, but not BAT volume was associated with BMI
Gatidis et al. [71]	NR	NR	102 (44)	58 ± 17	Patients	24 5	BMI	BAT+ participants had lower BMI than BAT-.
Brendle et al. [72]	2	-250/-50	602 (298)	49 ± 16	Patients	NR	BMI and CT	BAT+ participants had lower BMI than BAT-. BMI was negatively correlated to BAT, in different age-groups. BAT+ participants had lower subcutaneous fat mass than BAT-. Subcutaneous fat mass was inversely associated with BAT activity BAT+ participants had lower VAT and liver fat than BAT-. VAT was inversely associated with BAT activity.
Motiani et al. [73] *	NR	-250/-50	18 (0)	47 (95% CI: 43-49)	Healthy	25.3 (95%CI: 24.1-26.3)	BMI, MRI, BIA	BAT was negatively associated with BMI BMI was lower in high-BAT group than in low-BAT group. High-BAT group presented lower fat percentage than in low-BAT group BAT was negatively associated with WC VAT, subcutaneous fat mass and WC were lower in high-BAT group than in low-BAT group.
Puar et al. [74]	1,5	-180/-10	73 (33)	52.4 ± 15.4	Patients with pheochromocytomas and paragangliomas	25.2 ± 4.1	BMI	Participants with activated BAT presented a trend to have lower BMI than those with no BAT activation

Table 28. (Continued)

Study	SUV threshold	HU range	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m²)	BC meth	Results
No cold exposure: No association between BAT and whole-body adiposity								
Gerngroß et al. [75]	2	-250/-50	162 (108)	37.9 ± 17.1	NR	Case group: 22.7 ± 4.4 Control group: 22,8 ± 4.9	BMI	No differences in BMI between the BAT positive and BAT negative groups. BMI was not associated with the number of active BAT depots
Skillen et al. [76]	NR	NR	300 (117)	Warmed group: 57.7 (95% CI, 54.8–60.6), Non-warmed group: 61.0 (95% CI, 58.1–63.9).	Oncologic patients	Warmed: 27.1 (95% CI, 26.2–28.0) Non-warmed: 26.0 (95% CI, 25.1–26.9)	BMI	No significant differences were observed in BMI between BAT+ and BAT- participants
Choi et al. [77]	2	-250/-50	80 (80)	BAT+: 40 (36-42) BAT-: 42 (35-44)	Apparently patients	BAT+: 21.9 (20.4-23.8) BAT-: 21.9 (19.8-24.5)	BMI and DXA	No differences in BMI between BAT+ and BAT- participants. No differences in subcutaneous fat mass between BAT+ and BAT- participants. No differences in visceral fat mass between BAT + and BAT - participants
Miao et al. [78]	NR	-250/-50	45 (30)	BAT+: 35.67 ± 6.66 BAT-:37.43 ± 6.14	Healthy	BAT+: 20.12 ± 1.47 BAT-: 20.29 ± 1.43	BMI	No differences in BMI between BAT positive and control participants
Perkins et al. [79]	NR	-250/-50	386 (226)	NR	Evaluated for diagnostic reasons	NR	BMI	Neck/supraclavicular BAT prevalence was higher underweight individuals. Suprarenal BAT prevalence was higher in obese individuals.

Values are means ± standard deviation unless otherwise stated. NR: non-reported; SUV: standardized uptake value; thr: threshlold; HU: hounsfield unit; N: number; part: participants; BC: body composition; CI: Confidence interval; meth: method; BMI: body mass index; VAT: Visceral adipose tissue; WC: waist circumference; UCP-1: Uncoupling protein 1; BIA: bioelectrical impedance; DXA: Dual X-ray absorciometry. *: Indicates study where ^{18}F-FTHA was used in addition of ^{18}F-FDG.

Table 29. Human studies investigating the association the association of brown adipose tissue assessed with 18F-Fluordeoxyglucose (^{18}F-FDG) Positron Emission tomography (PET)-computerized tomography (CT) after a non-individualized cold exposure with body composition.

Study	SUV threshold	HU range	Cold-exposure	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m^2)	BC meth	Results
Non-personalized cold exposure; Negative relation between BAT and whole-body adiposity									
Bahler et al. [80]	2	-250/-50	Air 16-18°C	64 (0)	25.4 (21.4–31.8)	Healthy	22.9 (21.5–24.8)	BMI	BAT+ participants had lower BMI than BAT-. BAT+ participants had lower WC than BAT-.
Gifford et al. [81]	0	-200/-1	Air 17°C	17 (11)	25.1 ± 2.9	Apparently healthy	23.9 ± 2.9	BMI	BAT volume was inversely correlated to BMI. BAT volume was not associated with WC. associated with
Hibi et al. [82]	2	No	Air 19°C + intermittently placing a towel-wrapped ice block against the soles of their feet	21 (0)	26 ± 7	Apparently healthy	21.7 ± 1.6	BMI and DXA	BAT+ participants had lower BMI than BAT- (marginal differences). No differences in fat free mass between BAT+ and BAT-. No differences in fat mass between BAT+ and BAT- participants. No differences in fat mass percentage between BAT+ and BAT- participants.
Matsushita et al. [83]	2	NR	Air 19°C + feet in ice blocks	260 (74)	26 (p25= 22; p75=39)	Apparently healthy	21.6 (P25=20.1; P75=23.5)	BMI BIA and CT	BAT+ participants had lower BMI than BAT-. BAT+ participants had fat mass percentage BMI than BAT-. BAT+ participants had lower abdominal fat than BAT-.
Orava et al. [84] *	NR	NR	Air 17°C + intermittently placing of feet in cold water (8°C)	63 (45)	Lean: 39.6 ± 9.8 Obese: 38.1 ± 8.7	Apparently healthy	Lean: 22.7 ± 2.3 Obese: 34.0 ± 4.1	BMI	Lean subjects presented higher BAT glucose uptakes in response to cold and insulin, and higher blood flow in BAT during cold exposure. Participants with BAT positive had a lower BMI, waist circumference and body fat percentage.
Yoneshiro et al. [85]	2	NR	Air 19°C + intermittently placing the feet on an ice block	162 (59)	32.0 ± 12.1	Healthy	22.1 ± 3.0	BMI and BIA	BAT+ participants had lower BMI than BAT- participants. BAT+ participants had fat mass percentage BMI than BAT-. BAT+ participants had lower abdominal fat than BAT-. BAT+ participants had lower subcutaneous fat area than BAT-.

Table 29. (Continued)

Study	SUV threshold	HU range	Cold-exposure	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m²)	BC meth	Results
Van Marken Lichtenbelt et al. [1]	NR	NR	Air 16°C	24 (0)	Lean: 24.3±3.6 Overweight-obese: 23.5±3.4	Healthy	Lean: 23.2 ± 1.2 Overweight-obese: 30.3 ± 4.2	BMI and DXA	BAT activity was negatively associated to BMI. Lean individuals presented higher BAT activity than overweight-obese individuals, although a similar BAT volume. BAT activity was negatively associated with fat mass percentage.
Saito et al. [3]	NR	NR	Air 19°C + intermittently placing legs on an ice block	56 (25)	Men: 35.8 ± 9.0 Women: 38.8 ± 8.8	Apparently healthy	Men: 23.8 ± 2.6 Women: 21.1 ± 2.3	BMI and BIA	BAT (in BAT+) was inversely associated with BMI. BAT (in BAT+) was inversely associated with fat mass. BAT (in BAT+ participants) was inversely associated with Visceral adipose tissue. Body weight, BMI, and visceral and subcutaneous fat areas estimated from CT tended to be lower in the group bearing detectable BAT, but the difference was not statistically significant.
Yoneshiro et al. [86]	2	NR	Air 19°C + feet place on a ice block	260 (76)	26 (P25=22; P75=39)	Healthy	21.6 (P25=20.1; P75=23.5)	BMI, BIA, and CT	BAT + participants have a lower BMI than BAT -. BAT + participants have a lower body fat mass than BAT -. BAT + participants have a lower abdominal, visceral and subcutaneous fat area than BAT -. BAT, in addition to sex and age, in an independent determinant of BMI, body fat mass and abdominal visceral and subcutaneous fat areas.
Non-personalized cold exposure: No association between BAT and whole-body adiposity									
Yoneshiro et al. [87]	2	NR	Air 19°C + intermittently placing feet on an ice block	18 (0)	22.8 ± 0.7	Healthy	21.3 ± 0.4	BMI	No differences in BMI between BAT+ and BAT- participants.

Table 29. (Continued)

Study	SUV threshold	HU range	Cold-exposure	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m²)	BC meth	Results
Jang et al. [88]	1.5	NR	Air 19°C	17 (5)	36 ± 8	Healthy	25.4 ± 5.9	BMI	No differences in BMI between BAT+ and BAT- participants.
Orava et al. [89] *	NR	NR	Air 17°C	27 (20)	40.2 ± 9.4	Healthy	22.8 ± 2.2	BMI	No differences in BMI between BAT+ and BAT- participants.
Yoneshiro et al. [90]	2	-300/-10	Air 19°C + feet on ice block	15 (0)	23.1 ± 0.6	healthy	21.4 ± 0.7	BMI and BIA	No differences in BMI (neither weight) between BAT+ and BAT- participants. No differences in fat free mass between BAT+ and BAT- participants. No differences in fat mass between BAT+ and BAT- participants. No differences in WC between BAT+ and BAT- participants. No differences in fat mass percentage between BAT+ and BAT- participants.
Muzik et al. [91]	2	-250/-50	Air 16° C + fans to provide low-level airflow	14 (9)	30 ± 7	Apparently healthy	23.7 ± 2.8	BMI	No differences in BMI between BAT + and BAT- participants
Vrieze et al. [92]	2	-250/-50	Air 16-18°C	10 (0)	BAT+:24.5 (18–32) BAT-: 25.5 (21–29)	Apparently healthy	BAT+: 22.2 (20.3–24.0) BAT-: 22.2 (20.9–23.4)	BMI	No differences in BMI between BAT+ and BAT- participants.
Bahler et al. [93] †	NR	NR	Air 16-17°C	35 (0)	Young: 25.5 Old: 54	Healthy	Young lean: 22 Old men: 23.1 Obese young: 32.2	BMI	Metabolic BAT activity (maximal and mean) were different between the lean older men and lean young men but not between the obese and lean young men. The maximal sympathetic nervous stimulation of BAT (SQUVmax) and volume measured on 123I-mIBG SPECT were significantly diminished in the older men as compared with the lean young but did not differ between the obese and the lean young men. There was no correlation between the maximal or mean metabolic sympathetic BAT activity or BAT volume with BMI.

Table 29. (Continued)

Study	SUV threshold	HU range	Cold-exposure	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m²)	BC meth	Results
Bredella et al. [94]	70% SUVmax	-250/-50	Air 19°C + cooling vest at 17°c	15 (15)	Healthy: 25 ± 1.9 Anorexia: 30 ± 6.3 Anorexia recovered: 28.2 ± 7.3	5 anorexia nervosa patients, 5 recovered anorexia nervosa participants, and 5 healthy non-obese participants	Healthy 21.9 ± 1.4 Anorexia: 18.3 ± 0.9 Anorexia recovered: 22.4 ± 3.8	BMI, MRI, DXA	BAT was not associated with BMI BAT was not associated with fat free mass. BAT was not associated with fat mass. There was a trend of an inverse association between BAT and VAT.
Schlögl et al. [95]	2	-250/-10	Air 16°C	16 (7)	30.9 ± 9.9	Apparently healthy	26.4 ± 5.5	BMI and DXA	BAT was not associated with BMI BAT was negatively associated with fat free mass. BAT was not associated with fat mass. BAT was not associated with waist circumference. BAT was not associated with fat mass percentage.
Yoneshiro et al. [96]	2	NR	Air 19°C	51 (0)	24.4 ± 0.5	Healthy	22.0 ± 0.4	BMI and BIA	No differences in BMI between BAT+ and BAT- participants. No differences in fat free mass between BAT+ and BAT- participants. No differences in fat mass between BAT+ and BAT- participants. Of note is that the change in body fat mass was inversely associated with the change in BAT activity.
Muzik et al. [97]	2	-250/-50	Air 15.5°C + fans	25 (15)	Hihg BAT: 29.6 ± 5.5 Low Bat: 31.4 ± 9.7	Healthy	High BAT: 22.1 ± 3.1 Low BAT: 24.7 ± 3.9	BMI and skinfold measures	No differences in BMI between BAT+ and BAT- participants. Participants with high BAT had a lower lean body mass than participants with low BAT.
Nirengi et al. [98]	2	-300/-10	Air 19°C + intermittently placing feet on an ice block	29 (0)	23.3 ± 2.2	Healthy	21.6 ± 1.8	BMI and BIA	No differences in BMI between BAT + and BAT -. No differences in lean and fat mass between BAT + and BAT -.

Table 29. (Continued)

Study	SUV threshold	HU range	Cold-exposure	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m²)	BC meth	Results
Sugita et al. [99]	2	NR	Air 19°C + intermittently placing feet on an ice block	19 (0)	BAT+: 23.8 ± 1.1 BAT-: 21.3 ± 0.3	Apparantly healthy	BAT+: 21.6 ± 0.7, BAT-: 21 ± 0.4.	BMI and BIA	No differences in BMI between BAT+ and BAT- participants. No differences in fat free mass between BAT+ and BAT- participants. No differences in fat mass content between BAT+ and BAT- participants.
Yoneshiro et al. [100]	2	NR	Air 19°C + intermittently placing the feet on an ice block	45 (0)	23.4 ± 0.4	Healthy	21.7 ± 0.4	BMI and BIA	No differences in BMI between BAT+ and BAT- participants. No differences in fat mass between BAT+ and BAT- participants.
Sun et al. [101]	NR	2	Cooling vest 14.5°C	20 (12)	25.9 ± 0.9	Healthy	21.7 ± 0.6	BMI and DXA	No differences in BMI between BAT+ and BAT- participants. No differences in fat mass percentage between BAT+ and BAT- participants.
Non-personalized cold exposure: Positive association between BAT and whole-body adiposity									
Blondin et al. [18] ‡	1.5	-150/-30	Thermic suit 18°C	25 (0)	Healthy: 24 (22- 26) Age-matched controls: 59 (56- 62) Diabetic: 60 (56- 64)	Diabetic patients and controls	Healthy: 25.4 (23.6- 27.3) Age-matched controls: 26.3 (24.7- 28.9) Diabetic: 28.6 (21.5- 35.8)	BMI	BAT glucose uptake was not associated with BMI. Fractional and net NEFA uptake in BAT was positively associated with BMI. Glucose and NEFA uptake by BAT were not associated with fat mass. BAT glucose uptake was inversely associated with, WC but disappeared after adjusting by age. When adjusting by age, fractional and net NEFA uptake by BAT was positively associated with WC.
Singhal et al. [32]	1	-250/-10	Cooling vest 14°C	24 (24)	Athletes: 21.7 ± 2.2 Non-athletes: 21.6 ± 2.1	16 athletes and 8 non-athletes	Athlete: 21.8 ± 2.6 Non-athlete: 22.6 ± 2.4	BMI and DXA	BAT was not associated with BMI. BAT volume and BAT SUVmean were inversely correlated to lean mass BAT volume was positively correlated with fat mass. BAT volume was positively correlated with fat mass percentage.

Table 29. (Continued)

Study	SUV threshold	HU range	Cold-exposure	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m²)	BC meth	Results
Begaye et al. [102]	2	-250/-10	Air 16°C	12 (5)	31.10 ± 9.78	NR	26.03 ± 4.81	DXA	BAT SUVmean was negatively associated with fat free mass. BAT volume was positevly associated with fat mass. BAT volume was positively associated with fat mass percentage.
Lee et al. [30]	2	NR	Overnight (12 hours) mild cold exposure at 19°C	24 (11)	28 ± 7	Healthy	22.5 ± 2.1	BMI and DXA	No association between BAT parameters and lean body mass. There was no association between BAT parameters and body fat mass in men. BAT volume correlated positively with body fat mass in women, as it was BAT SUVmean (marginally).

Values are mean ± standard deviation unless otherwise stated. NR: non-reported; SUV: standardized uptake value; thr: threshlold; HU: hounsfield unit; N: number; part: participants; BC: body composition; meth: method; BMI: body mass index; VAT: Visceral adipose tissue; WC: waist circumference; UCP-1: Uncoupling protein 1; BIA: bioelectrical impedance; DXA: Dual X-ray absorciometry. *: Indicates study where [15O]H2O was used in addition of ^{18}F-FDG; †: Indicates study where 123I-mIBG was used in addition of ^{18}F-FDG; ‡: Indicates study where ^{18}F-FTHA and [11C]-acetate were used in addition of ^{18}F-FDG.

Table 30. Human studies investigating the association the association of brown adipose tissue assessed with 18F-Fluordeoxyglucose (^{18}F-FDG) Positron Emission tomography (PET)-computerized tomography (CT) after an individualized cold exposure with body composition.

Study	SUV threshold	HU range	Cold-exposure	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m²)	BC meth	Results
Personalized cold exposure; Negative relation between BAT and whole-body adiposity									
Hanssen et al. [103]	1.5	-180/-10	Individualized to shivering threshold	10 (0)	36.0 ± 13.0	Healthy	32.9 ± 3.5	BMI and DXA	BAT was negatively associated with fat mass percentage.
U Din et al. [50] *	NR	-250/-50	Individualized to shivering threshold	66 (45)	39.0 ± 9.8	Healthy	25.4 ± 4.4	BMI	BMI was lower in the high BAT group Cold-activated BAT radiodensity was negatively associated with BMI and WC.
Muzik et al. [104]	2	-250/-50	Individualized to shivering threshold	20 (10)	25.1 ± 5.2	Apparently healthy	23.0 ± 2.6	BMI and CT	BMI was lower in high-BAT participants than in low-BAT participants. Body fat percentage was similar between BAT+ and BAT- participants.
Leitner et al. [16]	Indiv.	-300/-10	Individualized to shivering threshold	20 (0)	Lean: 22.5 ± 4.9 Obese: 28.8 ± 4.7	Healthy	Lean: 23.2 ± 1.9 Obese: 34.8 ± 3.3	BMI	BAT volume and activity differences between lean and obese subjects were statistically significant when the individual SUVLBM thresholds were used, but not when the SUVTBM threshold was used. Lean participants seem to present a trend to have more active BAT than obese participants.
Personalized cold exposure; No relation between BAT and whole-body adiposity									
Chondronikola et al. [105]	1	-100/-10	Individualized to shivering threshold	12 (0)	BAT+: 41.2 ± 5.3 BAT- 49.8 ± 7.3	Healthy	BAT+: 28.2 ± 1.5 BAT-: 30.0 ± 2.8	BMI and DXA	No differences in BMI between BAT+ and BAT- participants. No differences in lean mass between BAT+ and BAT- participants. No differences in fat mass percentage between BAT+ and BAT- participants. No differences in subcutaneous adipose tissue mass between BAT+ and BAT- participants. No differences in visceral adipose tissue mass between BAT+ and BAT- participants.

Table 30. (Continued)

Study	SUV threshold	HU range	Cold-exposure	N participants (n women)	Age (years-old)	Health status	Body mass index (kg/m²)	BC meth	Results
Vijgen et al. [106]	NR	NR	Individualized to shivering threshold	15 (13)	39.2 ± 8.1	Morbidly obese subjects	42.1 ± 3.8 (34.8–48.3)	BMI and DXA	Only when merging data with other studies, BAT was negatively correlated to BMI. Only when merging data with other studies, BAT was negatively correlated with fat mass percentage.
Deng et al. [107] †	2	-200/-10	Individualized to shivering threshold	15 (0)	20.7 ± 1.5	Healthy	Normal-weight (6): (20.6–24.7) Overweight (5): (25.6–29.4) Obese (4): (31.3–35.9)	BMI and DXA	No association between BAT and BMI. No association between BAT and FM percentage. No association between BAT volume and fat mass ratio. Obese subjects showed higher fat fraction than nonobese subjects under all three thermal conditions.
Holstila et al. [108]	NR	NR	Individualized to shivering threshold	13 (6)	32.7 ± 9.9	Healthy	Normal weight (9). (20.3-24.7) Overweight (3) (25.0-31.5) Obese (1): 31.5	BMI	There was no association between BAT and BMI. There was no association between BAT and WC.
Lee et al. [109]	2	-300/-10	individualized to shivering	15 (2)	High BAT: 26 ± 2 Low BAT: 28 ± 2 Negative BAT: 25 ± 2	Apparently healthy	High BAT: 23 ± 2 Low BAT: 23 ± 2 BAT-: 23 ± 1	BMI and DXA	No differences in BMI between BAT positive and control participants. No differences in lean mass between BAT positive and control participants. No association of lean mass with BAT volume and activity. No differences in fat mass between BAT positive and control participants. No association of fat mass with BAT volume and activity.
Vijgen et al. [110]	NR	NR	Individualized to shivering threshold	10 (8)	40 ± 9	Morbid obese before and after bariatric surgery	Before surgery: 41.7 ± 4.4 After surgery: 29.8 ± 4.2	BMI and DXA	Before bariatric surgery, only 2 out of 10 participants showed active BAT. One year after surgery, the number of participants with active BAT was increased to five.

Values are mean ± standard deviation unless otherwise stated. NR: non-reported; SUV: standardized uptake value; thr: threshlold; HU: hounsfield unit; N: number; part: participants; BC: body composition; meth: method; BMI: body mass index; VAT: Visceral adipose tissue; WC: waist circumference; UCP-1: Uncoupling protein 1; BIA: bioelectrical impedance; DXA: Dual X-ray absorciometry; Indiv: individualized. *: Indicates study where ^{18}F-FTHA and [15O]O2 were used in addition of ^{18}F-FDG; †: Indicates study where MRI was used in addition of ^{18}F-FDG.

Table 31. Associations between brown adipose tissue (BAT) and body composition.

		BMI (kg/m²)			Lean mass (Kg)			Fat mass (Kg)			Fat mass (%)			VAT (g)		
		β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P
Model 1 (date)																
Ind-SUVt and SUV_{LBM}	BAT volume	0.020	0.055	**0.016**	0.015	0.008	0.376	0.038	0.055	**0.017**	0.025	0.037	0.059	0.784	0.055	**0.014**
	BAT metabolic activity	0.002	0.030	0.092	-0.001	0.001	**0.006**	0.006	0.042	**0.041**	0.005	0.054	**0.018**	0.090	0.025	0.099
	BAT SUVmean	-0.910	0.043	**0.036**	-2.050	0.047	**0.022**	-1.307	0.027	0.121	-0.145	0.005	0.835	-37.227	0.045	**0.026**
	BAT SUVpeak	-0.116	0.016	0.249	-0.341	0.025	0.098	-0.157	0.011	0.420	0.015	0.005	0.924	-5.295	0.018	0.172
Fix-SUVt and SUV_{BM}	BAT volume	0.024	0.094	**0.001**	0.011	0.005	0.488	0.052	0.117	**<0.001**	0.039	0.100	**0.001**	0.920	0.089	**0.001**
	BAT metabolic activity	0.003	0.040	**0.044**	-0.001	0.001	0.754	0.007	0.061	**0.012**	0.006	0.077	**0.004**	0.102	0.035	0.051
	BAT SUVmean	-0.375	0.025	0.132	-1.425	0.070	**0.005**	-0.179	0.007	0.711	0.576	0.024	0.145	-15.181	0.023	0.114
	BAT SUVpeak	-0.013	0.005	0.819	-0.226	0.034	0.053	0.068	0.009	0.540	0.171	0.037	0.058	-1.306	0.004	0.554
Model 2 (date and sex)																
Ind-SUVt and SUV_{LBM}	BAT volume	0.017	0.178	**0.029**	0.001	0.659	0.921	0.037	0.059	**0.021**	0.032	0.284	**0.006**	0.660	0.195	**0.025**
	BAT metabolic activity	0.003	0.173	**0.044**	0.000	0.659	0.834	0.006	0.051	**0.037**	0.005	0.273	**0.016**	0.101	0.188	**0.044**
	BAT SUVmean	-0.698	0.164	0.089	-1.064	0.671	**0.045**	-1.228	0.031	0.149	-0.613	0.240	0.322	-28.528	0.182	0.068
	BAT SUVpeak	-0.083	0.147	0.377	-0.193	0.666	0.113	-0.143	0.018	0.464	-0.053	0.234	0.707	-3.952	0.166	0.272
Fix-SUVt and SUV_{BM}	BAT volume	0.023	0.224	**0.001**	0.006	0.660	0.486	0.052	0.123	**<0.001**	0.041	0.338	**<0.001**	0.880	0.238	**0.001**
	BAT metabolic activity	0.003	0.187	**0.014**	0.001	0.659	0.667	0.007	0.071	**0.010**	0.005	0.290	**0.004**	0.118	0.201	**0.015**
	BAT SUVmean	-0.177	0.145	0.460	-0.519	0.667	0.091	-0.091	0.013	0.854	0.160	0.234	0.655	-6.990	0.162	0.443
	BAT SUVpeak	0.023	0.143	0.672	-0.068	0.661	0.331	0.086	0.018	0.444	0.101	0.244	0.213	0.157	0.157	0.939
Model 3 (date. sex and HOMA)																
Ind-SUVt and SUV_{LBM}	BAT volume	0.016	0.464	**0.012**	<0.001	0.718	0.991	0.035	0.347	**0.010**	0.030	0.441	**0.003**	0.628	0.457	**0.010**
	BAT metabolic activity	0.002	0.371	0.060	-0.001	0.134	0.691	0.005	0.337	**0.025**	0.005	0.156	**0.018**	0.083	0.350	0.063
	BAT SUVmean	-0.559	0.445	0.099	-0.931	0.727	0.057	-0.968	0.317	0.182	-0.465	0.398	0.404	-22.569	0.438	0.082
	BAT SUVpeak	-0.068	0.435	0.379	-0.178	0.725	0.112	-0.116	0.308	0.485	-0.039	0.395	0.759	-3.356	0.429	0.259
Fix-SUVt and SUV_{BM}	BAT volume	0.020	0.492	**0.001**	0.003	0.718	0.698	0.045	0.390	**<0.001**	0.037	0.480	**<0.001**	0.762	0.484	**0.001**
	BAT metabolic activity	0.002	0.378	**0.032**	-0.001	0.135	0.619	0.006	0.350	**0.007**	0.006	0.176	**0.004**	0.090	0.355	**0.036**
	BAT SUVmean	-0.188	0.436	0.339	-0.528	0.727	0.062	-0.119	0.306	0.778	0.137	0.395	0.671	-6.797	0.426	0.367
	BAT SUVpeak	0.010	0.431	0.822	-0.080	0.722	0.216	0.060	0.308	0.531	0.083	0.402	0.252	-0.266	0.422	0.876

Unstandardized β, R2, and P from multiple linear regressions. Model 1: Adjusted by date when BAT assessment was performed. Model 2: Model 1 and sex. Model 3: Model 2 and HOMA. BM: Body mass; BMI: Body mass index; fix-SUVt: Fixed SUV threshold (SUVt=2); ind-SUVt: Individualized SUV threshold [SUVt=1.2/(LBM/BM)]; LBM: Lean body mass; SUV: Standardized uptake value; VAT: Visceral adipose tissue

Figure 43 shows the associations between BAT mean activity (SUVmean) and body composition. We observed a trend for a negative association between BAT SUVmean and BMI (P=0.081), and a significant negative association between BAT SUVmean and VAT (P=0.047) (Figure 43), when assessing BAT with an individualized SUV threshold and SUV_{LBM}. The pattern of the association remained after adjusting by the date when the PET-CT was performed, sex, and HOMA (all P<0.1, Table 31). BAT SUVmean was not associated with fat mass or fat mass percentage (all P>0.1). BAT SUVmean was negatively associated with lean mass, both when assessed with an individualized SUV threshold and SUV_{LBM} (all P<0.037), or when assessed with a fixed SUV threshold and SUV_{BM} (all P<0.011). These

associations remained when adjusting by the date when the PET-CT was performed, sex, and HOMA (Table 31).

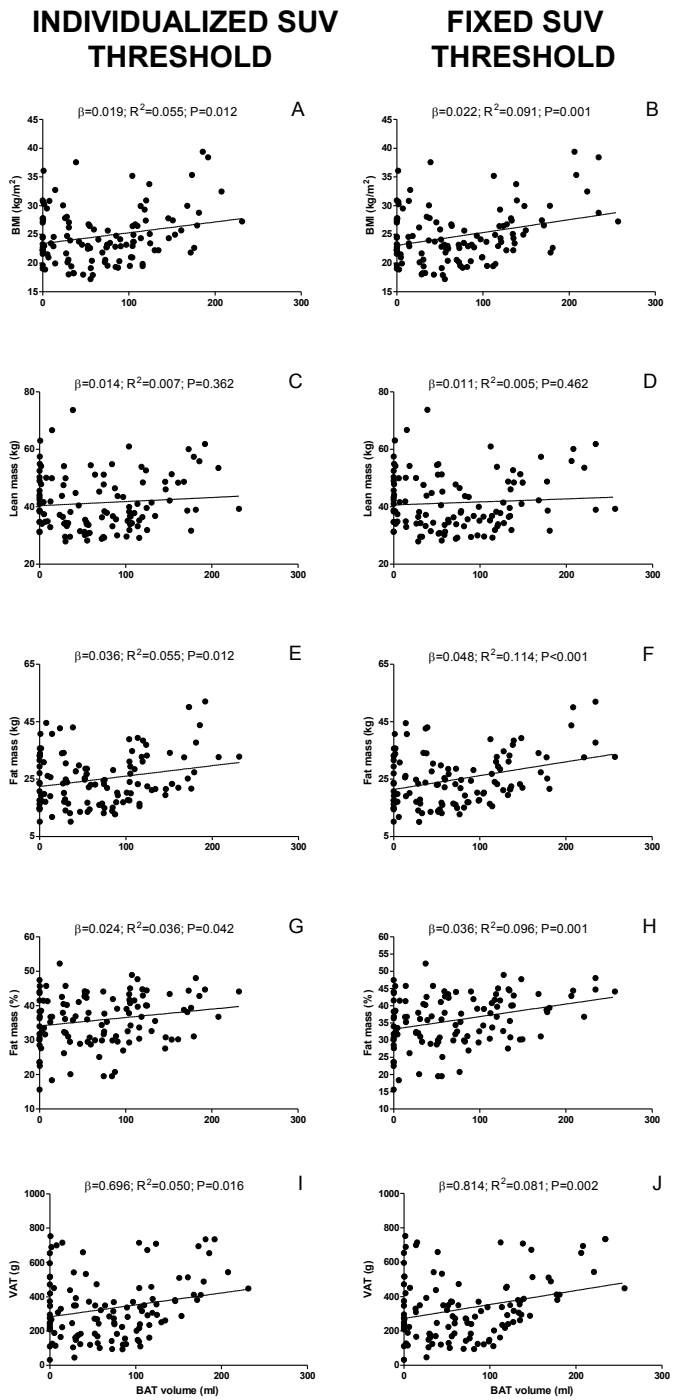

Figure 41. Associations between brown adipose tissue (BAT) volume and body composition. Unstandardized simple regression coefficient (β) and standardized coefficient of determination (R^2). Individualized standardized uptake value (SUV) threshold: [SUVt=1.2/(lean body mass/body mass)]; Fixed SUV threshold: (SUVt=2). BMI: Body mass index; VAT: Visceral adipose tissue.

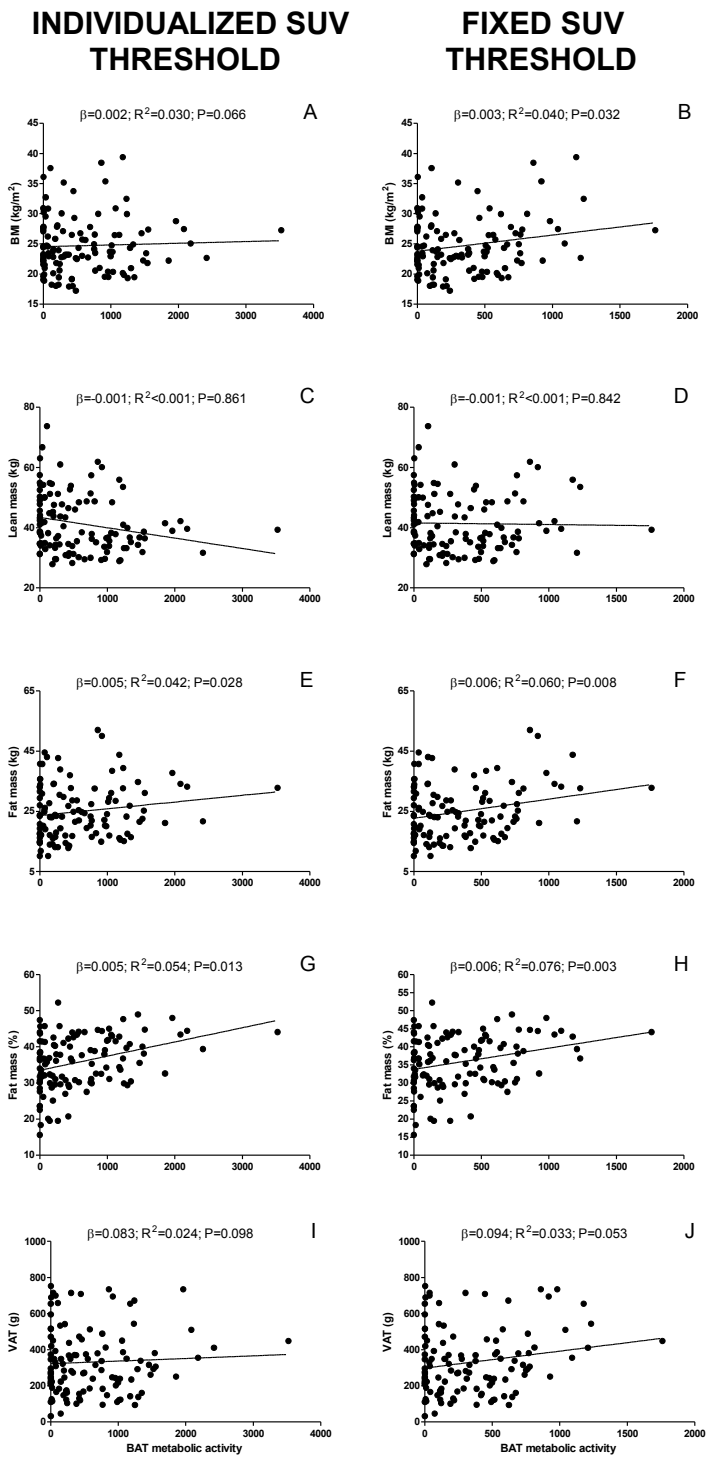

Figure 42. Associations between brown adipose tissue (BAT) metabolic activity and body composition. Unstandardized simple regression coefficient (β) and standardized coefficient of determination (R2). Individualized standardized uptake value (SUV) threshold: [SUVt=1.2/(lean body mass/body mass)]; Fixed SUV threshold: (SUVt=2). BMI: Body mass index; VAT: Visceral adipose tissue.

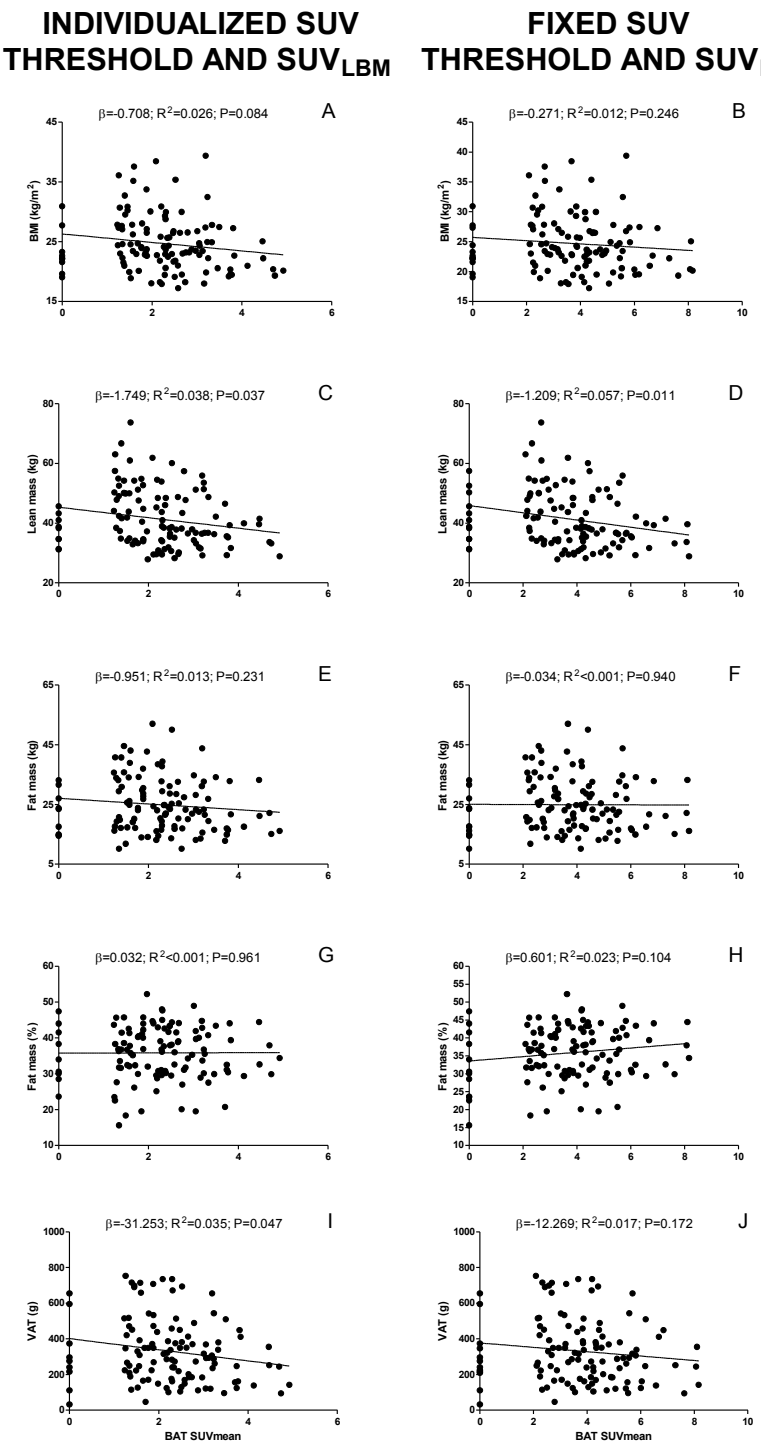

Figure 43. Associations between brown adipose tissue (BAT) mean activity and body composition. Unstandardized simple regression coefficient (β) and standardized coefficient of determination (R2). Individualized standardized uptake value (SUV) threshold: [SUVt=1.2/(lean body mass/body mass)]; Fixed SUV threshold: (SUVt=2).

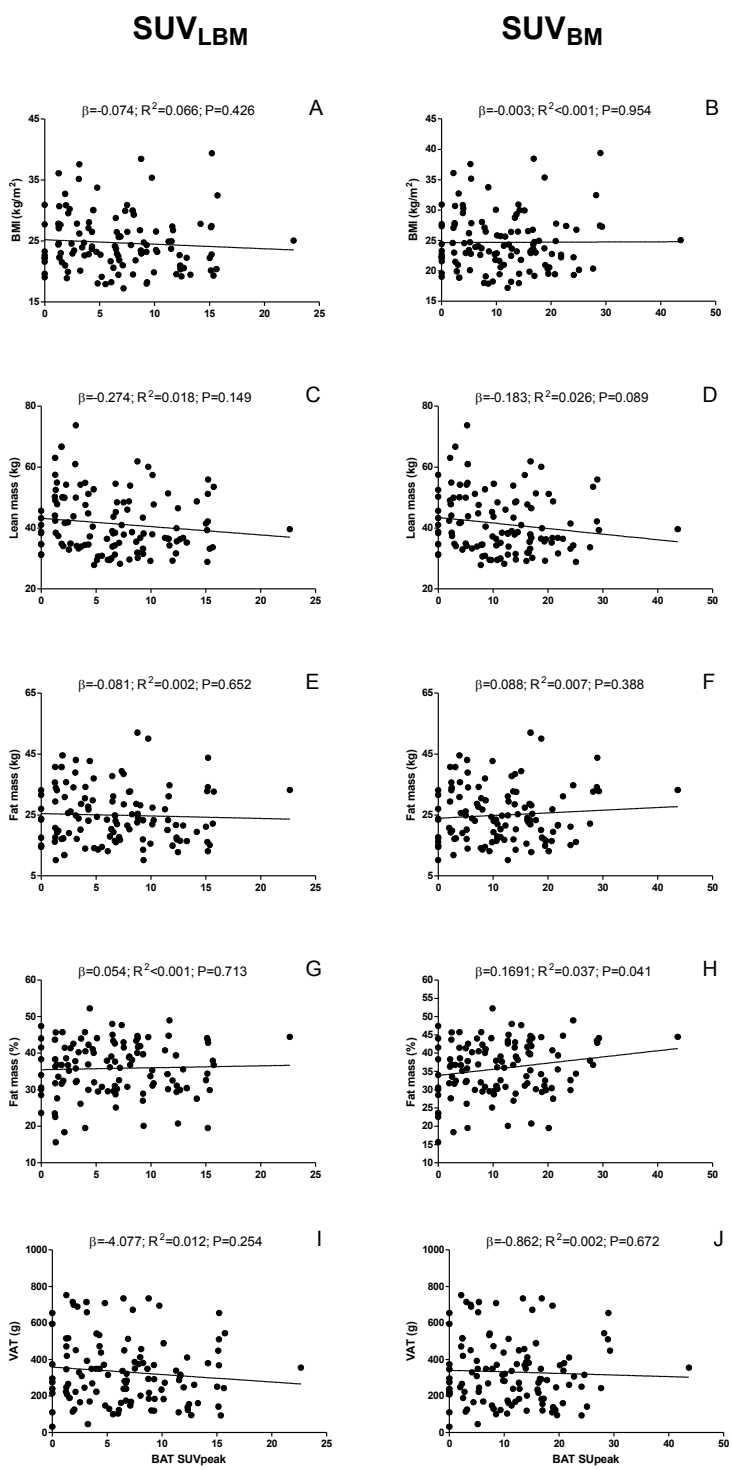

Figure 44. Associations between brown adipose tissue (BAT) peak activity and body composition. Unstandardized simple regression coefficient (β) and standardized coefficient of determination (R2). BMI: Body mass index; VAT: Visceral adipose tissue; SUV: Standardized uptake value.

Figure 44 shows the association between BAT maximal activity (SUVpeak) and body composition. When expressed as SUV_{LBM}, there were no association of BAT SUVpeak with any of the body composition variables (all P>0.14). When expressed as SUV_{BM}, BAT SUVpeak was positively associated to fat mass percentage (P=0.041), and marginally negatively associated with lean mass (P=0.081). However, both associations disappeared after adjusting by sex.

We additionally checked whether the observed significant associations with BAT volume or BAT activity (SUVmean) were dependent of the body surface area or BAT mean radiodensity, by replacing HOMA by either body surface area or BAT mean radiodensity in model 3, and all associations remained (data not shown).

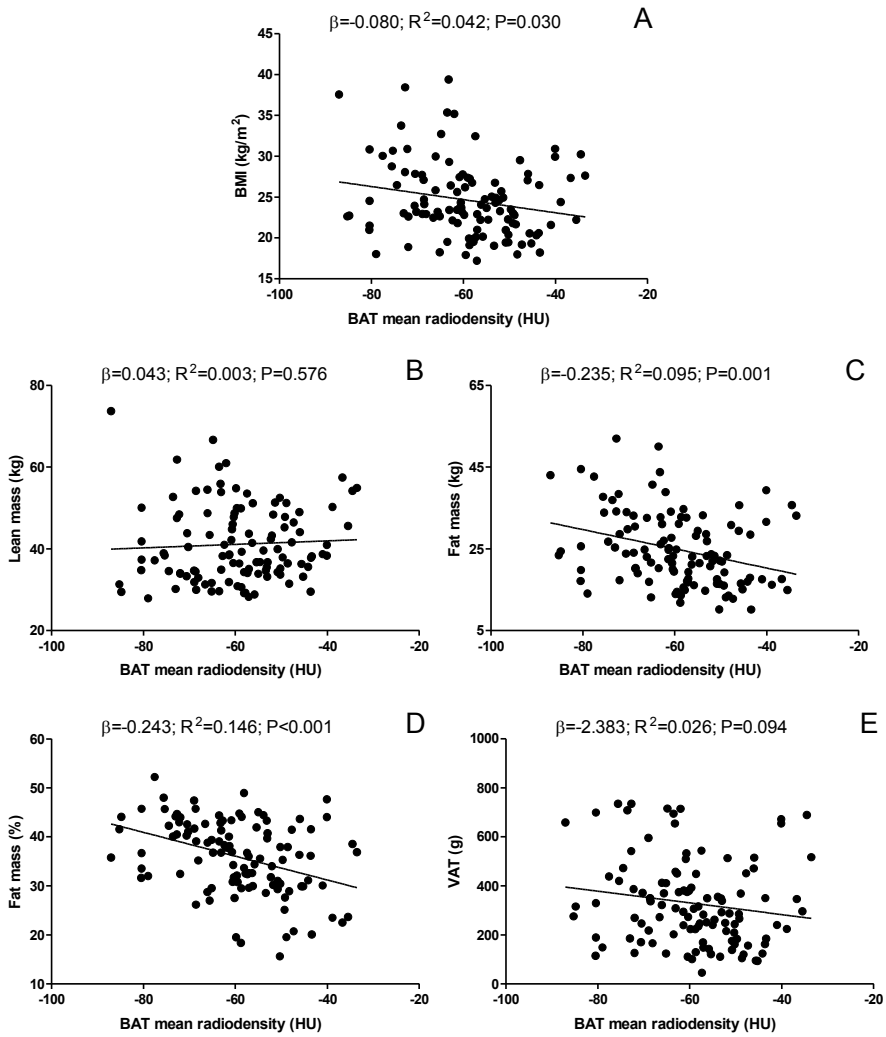

Figure 45. Associations between brown adipose tissue (BAT) mean radiodensity and body composition. Unstandardized simple regression coefficient (β) and standardized coefficient of determination (R2). BMI: Body mass index; VAT: Visceral adipose tissue.

Table 32. Associations of subcutaneous white adipose tissue (scWAT) and descending aorta ^{18}F-Fluorodeoxyglucose uptake with body composition.

		BMI (kg/m²)			Lean mass (Kg)			Fat mass (Kg)			Fat mass (%)			VAT (g)		
		β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P
Model 1 (date)																
SUV_{LBM}	scWAT upper back SUVpeak	3.439	0.018	0.254	12.683	0.039	**0.041**	4.478	0.013	0.438	-1.855	0.009	0.689	70.800	0.005	0.546
	scWAT tricipital SUVpeak	-7.612	0.011	0.409	66.329	0.111	**<0.001**	-35.652	0.041	**0.044**	-71.429	0.219	**<0.001**	-9.124	0.001	0.980
	Descending aorta SUVpeak	3.353	0.024	0.139	19.393	0.156	**<0.001**	1.824	0.007	0.678	-8.766	0.058	**0.014**	139.225	0.024	0.111
SUV_{BM}	scWAT upper back SUVpeak	3.924	0.049	**0.030**	5.727	0.022	0.129	8.526	0.062	**0.013**	5.156	0.038	0.063	113.095	0.025	0.109
	scWAT tricipital SUVpeak	2.831	0.006	0.656	42.324	0.095	**0.001**	0.342	0.005	0.978	-24.581	0.058	**0.013**	302.856	0.015	0.216
	Descending aorta SUVpeak	5.327	0.151	**<0.001**	7.751	0.074	**0.004**	11.531	0.189	**<0.001**	6.427	0.089	**0.002**	202.242	0.143	**<0.001**
Model 2 (date and sex)																
SUV_{LBM}	scWAT upper back SUVpeak	1.137	0.177	0.686	3.085	0.684	0.393	3.181	0.027	0.587	2.152	0.214	0.609	-22.411	0.178	0.837
	scWAT tricipital SUVpeak	-26.522	0.203	**0.004**	0.943	0.659	0.938	-50.414	0.073	**0.009**	-49.748	0.320	**<0.001**	-727.603	0.189	**0.041**
	Descending aorta SUVpeak	-0.408	0.141	0.861	3.369	0.662	0.264	0.212	0.013	0.965	-1.461	0.234	0.676	-15.280	0.157	0.864
SUV_{BM}	scWAT upper back SUVpeak	3.360	0.207	**0.043**	3.330	0.689	0.120	8.223	0.075	**0.017**	6.170	0.256	**0.013**	90.566	0.193	0.161
	scWAT tricipital SUVpeak	-5.073	0.146	0.415	9.696	0.663	0.227	-3.281	0.013	0.799	-10.260	0.241	0.270	-3.871	0.157	0.987
	Descending aorta SUVpeak	4.674	0.251	**<0.001**	4.465	0.682	**0.005**	11.438	0.190	**<0.001**	8.139	0.366	**<0.001**	175.042	0.261	**<0.001**
Model 3 (date. sex and HOMA)																
SUV_{LBM}	scWAT upper back SUVpeak	3.163	0.492	0.167	5.096	0.756	0.122	7.329	0.350	0.140	4.855	0.397	0.204	27.207	0.451	0.764
	scWAT tricipital SUVpeak	-27.200	0.497	**<0.001**	0.254	0.718	0.982	-51.578	0.368	**<0.001**	-50.310	0.484	**<0.001**	-749.571	0.457	**0.010**
	Descending aorta SUVpeak	-1.376	0.434	0.475	2.474	0.720	0.373	-1.669	0.306	0.685	-2.620	0.398	0.405	-45.376	0.424	0.538
SUV_{BM}	scWAT upper back SUVpeak	3.665	0.519	**0.006**	3.586	0.759	0.066	8.996	0.395	**0.002**	6.803	0.440	**0.002**	85.943	0.464	0.108
	scWAT tricipital SUVpeak	-10.423	0.453	**0.042**	4.907	0.719	0.511	-13.413	0.315	0.223	-16.493	0.416	**0.049**	-194.823	0.427	0.324
	Descending aorta SUVpeak	2.800	0.468	**0.008**	2.717	0.726	0.076	7.985	0.385	**<0.001**	6.110	0.464	**<0.001**	109.944	0.461	**0.006**

Unstandardized β, R^2, and P from multiple linear regressions. Model 1: Adjusted by date when BAT assessment was performed. Model 2: Adjusted by date when BAT assessment was performed and sex. Model 3: Adjusted by date when BAT assessment was performed, sex and HOMA. BM: Body mass; BMI: Body mass index; LBM: Lean body mass; SUV: Standardized uptake value; VAT: Visceral adipose tissue.

Figure 45 shows the association between BAT mean radiodensity and body composition. BAT mean radiodensity was negatively associated with BMI, total fat mass and fat mass percentage (all P<0.003), whereas the negative association between BAT mean radiodensity and VAT did not reach statistical significance (P=0.094). Lean mass was not associated with BAT mean radiodensity (P=0.576). These results did not change after adjusting by the date when the PET-CT was performed, sex, and HOMA (data not shown).

Table 32 shows the associations of subcutaneous white adipose tissue and descending aorta ^{18}F-FDG activity with body composition. Subcutaneous adipose tissue SUVpeak in the tricipital area was negatively associated to BMI and adiposity when expressed as SUV_{LBM} after adjusting by the date when the PET-CT was performed, sex and HOMA (all P<0.010). We observed a similar pattern, yet not reaching statistical significance, when subcutaneous adipose tissue SUVpeak was expressed as SUV_{BM} (P<0.224). Descending aorta ^{18}F-FDG activity was consistently associated with all variables of body composition when expressed in SUV_{BM} and only with lean mass when expressed as SUV_{LBM}, although this association disappeared after adjusting by sex (Table 32).

DISCUSSION

This is the first study showing a positive association of BAT volume, assessed by ^{18}F-FDG PET-CT after a personalized cold-exposure and strictly following the current recommendations [13], with whole-body and central adiposity in young healthy adults. This is in contrast with most previous reported human studies. However, it should be noted that most of these studies were conducted with PET-CT analysis methodologies that were not standardized to the recent recommendations for BAT assessment, and more importantly, not individualizing cold exposure before the PET-CT (see tables 28-30) [13]. To highlight is that our study is, by far, the largest to have assessed BAT strictly following current methodological recommendations[13]. Moreover, our study sample was comprised by young (18-25 years) healthy adults, which precludes ^{18}F-FDG uptake being biased by insulin resistance (of note, adjusting by HOMA did not modify any association), as could occur in older individuals. These findings concur with the already well-established animal physiology principles showing BAT growth in response to high-fat diet-induced body weight increases[4], and with recent human studies suggesting a negligible role of BAT on human EE [23–27].

The association between BAT, assessed by ^{18}F-FDG-PET-CT, and body composition has been reported in a high number of studies during the last decade (tables 28-30). Currently, it is well accepted that performing a ^{18}F-FDG PET-CT after a personalized cold exposure is the gold-standard for in vivo human BAT quantification [13,17,28], and that not individualizing cold exposure before the PET-CT likely could result in over or underestimation of BAT, representing a risk of bias, especially when comparing participants with different body composition [13,14]. However, only 15% (n=10) of the studies investigating the association between BAT and body composition performed an individualized cold exposure prior to the PET-CT. Furthermore, among the studies applying a personalized cold exposure, only one used an individualized SUV threshold and SUV_{LBM} [29]. Importantly, the observed association in our study between the reference tissue (i.e. descending aorta) ^{18}F-FDG activity and body composition when using SUV_{BM} but not when using SUV_{LBM}, are in agreement with Leitner et al. [29] and further reinforce the need of using SUV_{LBM}. Although it has been widely assumed that BAT volume is negatively associated with whole-body adiposity in humans, it is important to consider that most of the studies applied methodologies that could bias the relation between BAT and body fat, either because the lack of a personalized cold exposure before the ^{18}F-FDG PET-CT (84.6% of studies), or because they used a non-individualized SUV threshold and SUV_{BM} (98.5% of studies). Furthermore, the radiodensity criteria to define BAT importantly impact BAT estimations [17], and most of the studies investigating the relation between BAT and body fat were published before a consensus was reached regarding the appropriate radiodensity range [13]. Moreover, it should be noted that the reported BAT-fat negative association has only been shown by less than a half of the available studies.

Our findings of a positive relation between BAT volume and different markers of whole-body and central adiposity are therefore in contrast with most of previously published studies (tables 28-30). Nonetheless, it is notable that the positive association between BAT and adiposity remained when analysing the PET CT using a fixed SUV threshold and using SUV_{BM}. The maintenance of the associations even when using the older analysis parameters suggests that the age of the participants and/or the use of a

personalized cold exposure protocol as the most plausible reasons for explaining the discrepancy with most of scientific literature. Indeed, as expected, BMI was positively correlated with the time to shivering (i.e. inversely correlated with the water temperature when shivering occurred) (β=2.371; R^2=0.094; P=0.001). However, of note, the time to shivering was not associated with BAT volume and SUV mean (both P>0.1) and was even negatively associated with BAT SUVpeak (β=-0.823; R^2=0.037; P=0.045), discarding the possibility of the lower temperature exposure in participants with higher BMI as an explaining factor of the association between BAT volume and whole-body adiposity.

Others have previously reported positive associations between BAT volume/activity and adiposity in small samples [18,30–33], although no study applying a personalized cold exposure before the PET-CT has previously shown the positive association between BAT and body adiposity (table 30). Moreover, besides the studies assessing BAT volume by ^{18}F-FDG PET-CT, our results are also partially in contrast with studies assessing BAT using histology [34] or that have analysed UCP-1 gene expression [35,36]. Obviously, these discrepancies cannot be explained by PET-CT methodological issues or the cooling protocol. Therefore, the age of the participants seems to be the only clearly distinctive characteristic between our study and most of the previous ones. Thus, it might be that the negative relation between BAT and adiposity, either assessed histologically, by UCP-1 expression, or by ^{18}F-FDG uptake is indeed mediated by the age of participants. It has been repeatedly reported that aging decreases BAT volume and activity [2,22,37–39], and aging is also commonly associated with a progressive increase in whole-body and central adiposity, as well as with insulin resistance development [40,41]. Therefore, it might be that the reported lower BAT volume observed in obese individuals could actually be real in middle-age adults but not in young adults, since aging increases both whole-body and central adiposity, and insulin resistance but also reduces BAT volume [2]. Alternatively, it could be that increasing fat mass drives an increase in BAT volume in young ages, but if sustained over time, impairs BAT function and expandability, which would explain the inverse association between BAT and adiposity in older individuals. Age-related insulin resistance may also be accompanied by catecholamine resistance [42–45]. Thus, it could be that because most of the young overweight or obese individuals in our study did not present with significant insulin resistance, we have observed higher BAT volume in participants with higher levels of fat mass, which after many years of sustained obesity could be reversed because of factors such as insulin and catecholamine resistance [27,46,47].

We observed a negative association between BAT radiodensity (a proxy of fat content, i.e. the lower the radiodensity the higher the fat content) and body adiposity. Importantly, the association between BAT volume and body fat was still present when accounting for BAT radiodensity, suggesting that increases in BAT volume were not purely driven by lipid accumulation causing brown adipocyte hypertrophy. Therefore, the higher BAT volume and BAT total glucose uptake (i.e. BAT metabolic activity) and the higher BAT fat content (assessed by radiodensity) suggest both BAT hypertrophy (i.e. increased brown adipocytes size driven by increased fat content) and BAT hyperplasia (i.e. the recruitment of new brown adipocytes) may occur in parallel to body fat increase.

The hypothesis of BAT recruitment driven by increases in fat mass is in agreement with earlier studies showing higher UCP-1 expression in the subcutaneous [48] and epicardial [49] fat of obese patients, and with murine studies [5–9]. Nonetheless, if it is assumed that fat

gain induces BAT hypertrophy and/or hyperplasia in humans, its physiological relevance remains to be determined. The increase in BAT volume induced by fat mass gain hypothesis is in agreement with the mechanism first proposed by Rothwell and Stock [4]. They showed that BAT is recruited in order to increase adaptive thermogenesis for counteracting excess positive energy balance. However, the hypothesis of obesity-induced thermogenesis would be in contrast with the lack of association that we observed between body adiposity and BAT SUVmean and SUVpeak, and with previous studies showing that BAT's contribution to EE in humans is rather low [23,24,26,27]. Moreover, it seems that BAT fat content is negatively associated with BAT thermogenesis in humans [28,50], and therefore, the negative association observed between BAT radiodensity and whole-body adiposity suggest that despite having higher BAT volume, obese patients present a lower BAT thermogenic capacity. Alternatively, both the increased BAT volume and the higher BAT fat content observed in individuals with greater adiposity in our study would be compatible with the idea of a parallel expansion of WAT and BAT. A failure in WAT expansion seems to be the critical factor in determining when lipotoxicity occurs in other organs, and therefore in the development of the metabolic syndrome [51]. BAT expandability could also combat the onset of the metabolic complications of obesity partly by serving as small additional fat store, preventing ectopic lipid accumulation, but also by reducing blood lipid levels by oxidising fats and due to its role as an endocrine organ[52], that can produce insulin sensitizing hormones. Therefore, our data support the hypothesis of BAT expandability being parallel to WAT expandability and suggest this may be a factor in the development of metabolic abnormalities in the context of obesity. Indeed, the negative relation between BAT SUVpeak and VAT despite a positive association between BAT volume and VAT might indicate that BAT activity is protective against central fat accumulation, therefore protecting against the development of metabolic abnormalities.

Besides the association between BAT volume and whole-body and central adiposity, we also observed trends to negative associations between BAT SUV mean and lean mass, and between BAT SUVpeak and VAT. NST in humans is produced not only by BAT, but also by skeletal muscle activity [27], with skeletal muscle reported to be the main contributor [23]. Moreover, it has been shown that the relation between BAT and muscle thermogenesis is modified by cold acclimation [53]. Therefore, it could be that individuals with higher lean mass would have a higher contribution of skeletal muscle (or even other lean tissues) to thermogenesis, and consequently presents a lower BAT activity, although this hypothesis needs to be tested in future studies.

The results of this study should be considered with caution as some limitations arise. First, the cross-sectional design precludes to stablish causal relationships. Moreover, the study sample was entirely comprised of young adults, and we do not know whether these findings apply to older individuals. Finally, although the ^{18}F-FDG PET-CT is nowadays considered the gold standard to assess BAT volume in vivo, this method has some limitations (e.g. it does not allow the observation of small brown fat depots spread within WAT). Consequently, this study needs to be replicated once a new technology for BAT assessment in vivo has been developed.

In summary, this study shows that BAT volume, but not its glucose uptake capacity (SUVmean and SUVpeak), is positively associated to whole-body and central adiposity in young adults, which is consistently detected with different SUV thresholds. Moreover, we

also observed a positive association between BAT fat content and whole-body and central adiposity in young adults. These results, together with the conducted systematic review, suggest that the widely extended belief of that BAT volume is reduced in obese individuals seems to be wrong, at least in young individuals. These findings open the possibility of considering BAT expandability as a key factor for counteracting metabolic abnormalities development in the context of obesity, instead of considering it a target for weight management.

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SECTION 3

ROLE OF BROWN ADIPOSE TISSUE AND ENERGY BALANCE REGULATION IN THE DEVELOPMENT OF THE METABOLIC SYNDROME

Brown adipose tissue, whole-body adaptive thermogenesis and metabolic flexibility in metabolically healthy overweight-obese adults and in metabolically unhealthy counterparts: a case-control study

STUDY 9

BACKGROUND

Obesity has increased markedly over the last decades and it is considered to be a pandemic [1]. Obesity is a pathological condition associated with higher risk of suffering cardio-metabolic alterations as well as a myriad of other physical and psychological problems [1]. However, there is a subset of obese individuals characterized by a lower risk of obesity-related cardio-metabolic complications [2,3], the so called MHO phenotype. MHO individuals are obese but do not have dyslipidaemia, hyperglycaemia, or hypertension [2,3] and have a lower risk of developing type 2 diabetes and cardiovascular diseases [4–8]. Moreover, MHO individuals present lower risk of heart failure compared with normal-weight individuals with metabolic syndrome [9]. Estimates indicate that one in every three obese are MHO [10], although most of MHO individuals become MUO later in time [11,12]. Importantly, the proportion of MHO becoming MUO is higher than the proportion of metabolically healthy normal-weight individuals who develop the unhealthy phenotype [11], suggesting that obesity amplifies the aging-induced metabolic impairments [13]. Unravelling the physiological mechanism behind the intriguing MHO phenotype could be a source of solutions in the prevention of metabolic and cardiovascular diseases.

In humans BAT´s direct contribution to whole-body EE is much more modest than in small mammals [14–17]. Nonetheless, either because BAT could indirectly orchestrate adaptive thermogenesis [14,18], or because its endocrine activity could promote lipid lowering and insulin sensitizing effects [19,20], BAT is still considered a promising target for metabolic and cardiovascular disease prevention and treatment [21]. Therefore, it is biologically plausible that MHO individuals have higher BAT activity and volume than their MUO counterparts.

BAT was thought to play an important role in human´s adaptive thermogenesis [22,23] and metabolic flexibility [15,24]. Adaptive thermogenesis is defined as the increase in EE above basal levels to preserve body temperature, either in response to cold exposure or to a meal ingestion [22,25]. Metabolic flexibility refers to the adaptive response of an organism's metabolism to maintain energy homeostasis by matching fuel availability and demand to conditional changes such as periodic fasting, varying meal composition, physical activity, or environmental fluctuation [26,27]. Both adaptive thermogenesis and metabolic flexibility seem to play an important role in the prevention and development of metabolic and cardiovascular diseases [26–28]. Therefore, it is also biologically plausible that MHO individuals have higher adaptive thermogenesis and metabolic flexibility than their MUO counterparts.

Of note, when classified by BMI not only obese, but also over-weight people is prone to develop metabolic abnormalities [29]. In the present study we tested whether MHOO individuals present different BAT volume and activity, whole-body adaptive thermogenesis and metabolic flexibility compared than MUOO young adults.

METHODS OVERVIEW

General overview

A total of 53 young adults (18-25 years old, 52.8% females) participated in this study (Table 33). Participants were retrospectively selected among the participants of the ACTIBATE study. Participants were classified as MHOO or MUOO [30]. MUOO was defined as having a BMI ≥ 25 kg/m^2 and presenting at least one of the following criteria: a) Serum triglyceride concentration ≥ 150mg/dL; b) HDL concentration <40 mg/dL for men and 50 mg/dL for women; c) Systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg; d) Serum glycaemia >100 mg/dL. MHOO was defined as having a BMI ≥ 25 kg/m^2 and no presenting any of the above-mentioned risk factors. Table 34 shows the methodology overview. For a more detailed methods description see "Methods" section.

Table 33. Descriptive characteristics of metabolically healthy overweight-obese (MHOO) and metabolically unhealthy overweight-obese (MUOO) participants.

	ALL (n=53; 52.8% women)		MHOO (n=34; 68% women)		MUOO (n=19; 26% women)	
Age (years)	22.20	(2.38)	21.82	(2.35)	22.89	(2.34)
Weight (kg)	86.80	(15.04)	83.07	(12.32)	93.45	(17.37)
Height (m)	1.70	(0.09)	1.69	(0.08)	1.73	(0.10)
BMI (kg/m^2)	29.75	(3.55)	28.97	(3.35)	31.14	(3.56)
Lean mass (kg)	47.51	(10.04)	45.17	(8.66)	52.33	(11.22)
Fat mass (kg)	33.46	(7.14)	33.08	(7.22)	34.23	(7.15)
Fat mass (%)	40.24	(6.38)	41.07	(6.86)	38.54	(5.03)
VAT mass (g)	499.08	(147.22)	462.35	(145.92)	574.85	(121.84)
Glucose (mg/dl)	90.32	(7.89) †	87.59	(6.48)	95.21	(7.97)
Insulin (µUl/ml)	11.79	(7.82) †	9.37	(4.58)	16.14	(10.36)
HOMA	2.74	(2.16) †	2.07	(1.13)	3.96	(2.95)
HDL (mg/dl)	48.26	(9.89) †	51.21	(9.14)	43.00	(9.15)
LDL (mg/dl)	97.72	(70.82) †	90.74	(17.49)	112.53	(38.74)
TC (mg/dl)	164.75	(34.21) †	155.35	(21.61)	181.58	(45.39)
TG (mg/dl)	98.55	(28.72) †	67.24	(22.99)	152.26	(92.87)
SBP (mmHG)	122.41	(12.19) †	116.82	(8.35)	132.11	(11.86)
DBP (mmHG)	73.91	(7.33) †	71.25	(6.03)	78.52	(7.23)

Data are means and standard deviation; BMI: Body mass index; VAT: Visceral adipose tissue; 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. P for group comparisons (un-paired t-test). †: P<0.05 from analysis of covariance adjusting by sex to test MHOO vs. MUOO differences.

Statistical analyses

Results are presented as means ± SD, unless otherwise stated. The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 21.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set at <0.05.

We compare 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=23, 68%; n=5, 26%, respectively, P=0.004, Table 33), all the subsequent analyses were adjusted by sex.

Table 34. Study 9 methodology.

GENERAL INFORMATION	
General aim	To compare BAT and skeletal muscle ^{18}F-FDG act. and energy balance related variables between MHOO and MUOO
Design	Case-control
Cohort and participants	ACTIBATE (n=53)

OUTCOMES INCLUDED IN THE STUDY	
Outcome	Variables included
CVD	Serum glucose Serum HDL Serum triglycerides Blood pressure
BCa	BMI
PET-CT	BAT volume (-190/-10 HU; Ind. SUV threshold) BAT metabolic activity (-190/-10 HU; Ind. SUV threshold) BAT SUVmean (-190/-10 HU; Ind. SUV threshold) BAT SUVpeak (-190/-10 HU; Ind. SUV threshold) BAT mean radiodensity All muscles SUVpeak Deep muscles SUVpeak Cervical muscles SUVpeak Cold sensitive muscles SUVpeak Tricipital subcutaneous WAT SUVpeak Descending aorta SUVpeak
BIC	BMR Basal FATox
PPIC	MIT Meal-induced RER changes Meal-induced CHOox Meal-induced FATox
CEIC	CIT Cold-induced RER changes
APP	Ad libitum energy intake Energy intake estimated by 24 hours dietary recalls Hungry sensation Fullness sensation
MFOa	MFO Fatmax

BAT: Brown adipose tissue; ^{18}F-FDG: ^{18}F-Fluorodeoxyglucose; MHOO: Metabolically healthy overweight or obese; MUOO: Metabolically unhealthy overweight or obese; CVD: Cardiovascular disease risk; HDL: High-density lipoprotein cholesterol; BCa: Body composition assessment; BMI: Body mass index; PET-CT: Positron emission tomography-Computerized tomography; Ind: Individualized; SUV: Standardized uptake value; BIC: Basal indirect calorimetry; BMR: Basal metabolic rate; FATox: Fat oxidation; PPIC: Post-prandial indirect calorimetry; MIT: Meal-induced thermogenesis; RER: Respiratory exchange ratio; APP: Energy intake and appetite regulation;CHO: Carbohydrates oxidation; CIT: Cold-induced thermogenesis; MFOa: Maximal fat oxidation assessment; MFO: Maximal fat oxidation during exercise; Fatmax: the intensity at which MFO is reached.

We used ANCOVA adjusting by sex, to compare BAT, skeletal muscle and descending aorta glucose uptake, BAT radiodensity, BMR, resting FATox, MIT, CIT, MFO

and Fatmax between MHOO and MUOO. In sensitivity analyses, we further included BMI or HOMA as potential confounders (covariates) in the models, since both body weight and insulin resistance are related to BAT volume and activity [31], adaptive thermogenesis [28] and metabolic flexibility [26].

We also used bifactorial mixed ANCOVA adjusting by sex, to test differences between MHOO and MUOO on the kinetics of EE, RER and nutrient oxidation rates after the meal (MIT) and during cold exposure (CIT).

RESULTS

Figure 46 shows representative ^{18}F-FDG PET-CT images from two individuals. BAT volume, metabolic activity, SUVmean, and SUVpeak were significantly higher in MHOO than in MUOO (2.24, 2.45, 1.62, and 2.23-fold higher respectively, all P<0.017, Figure 47A-D, respectively). These findings remained after adjusting by BMI (all P<0.016) or HOMA (all P<0.007). Mean BAT radiodensity was similar between groups (P=0.564; Figure 48).

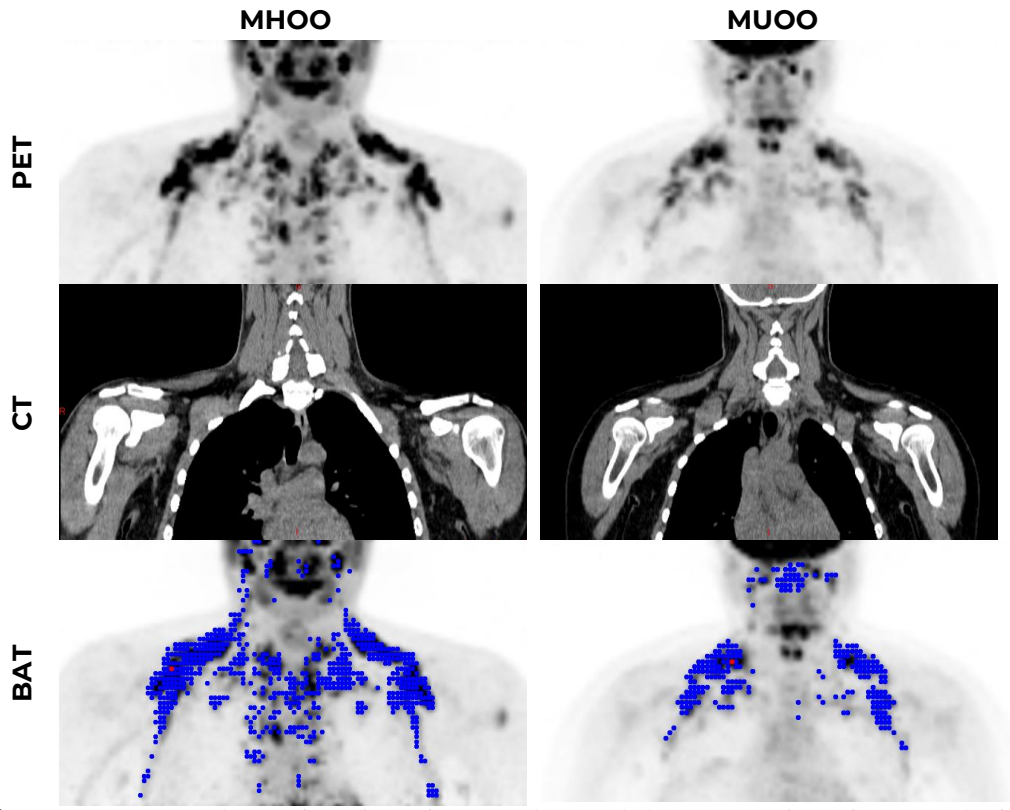

Figure 46. Representative ^{18}F-Fluorodeoxyglucose positron emission tomography and computerized tomography images from two individuals, one metabolically healthy overweight-obese (MHOO) and another one metabolically unhealthy overweight-obese (MUOO). Blue dots indicate brown adipose tissue (BAT) and read dots indicate maximal BAT activity (maximal standardized uptake value). PET: Positron emission tomography; CT: Computerized tomography.

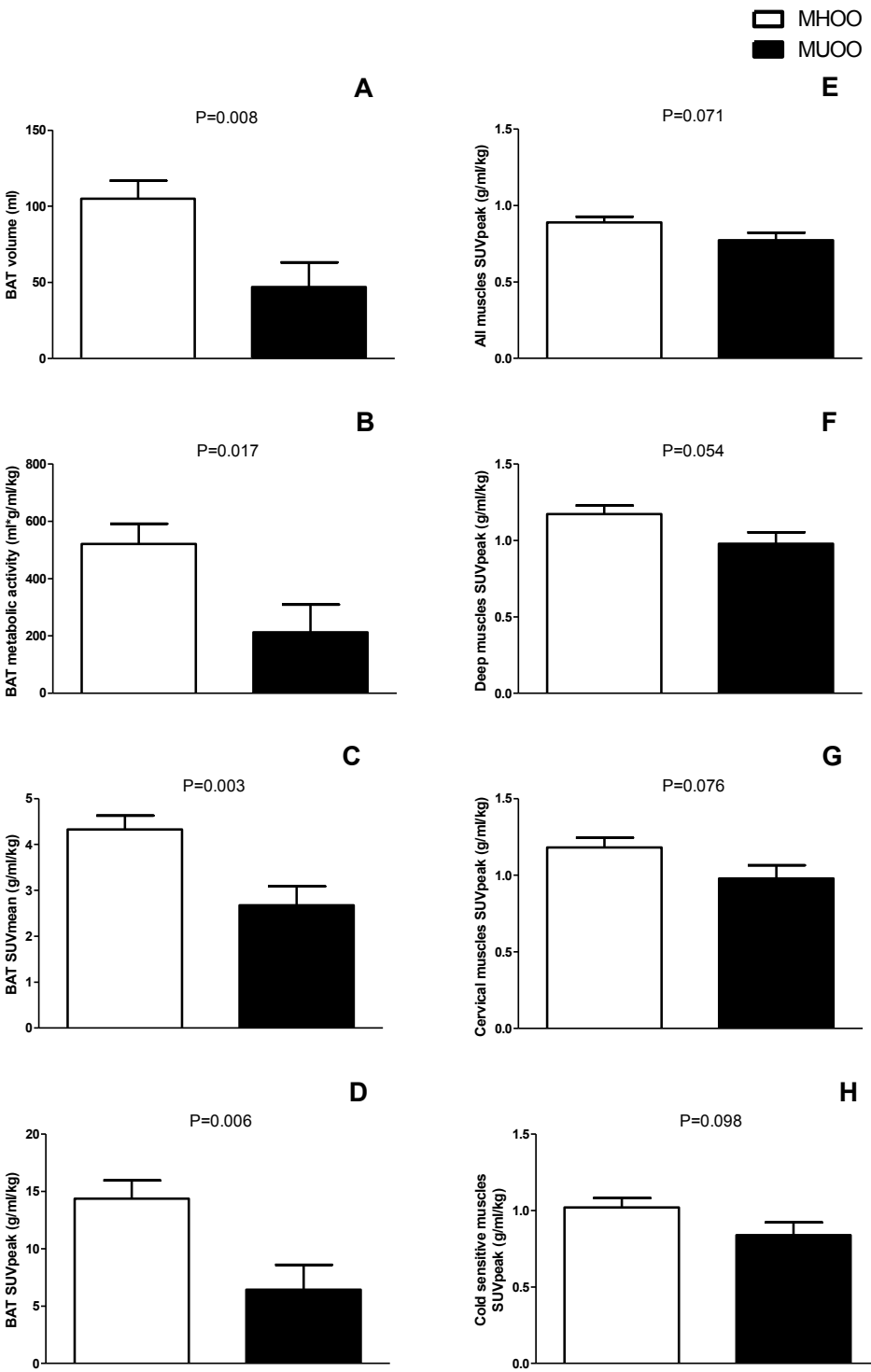

Figure 47. Brown adipose tissue (BAT) and skeletal muscle glucose uptake in metabolically healthy overweight-obese (MHOO) (n=34) and metabolically unhealthy overweight- obese (MUOO) (n=19) participants. Dara are adjusted means and standard error. P from analysis of covariance adjusting by sex. SUV: Standardized uptake value.

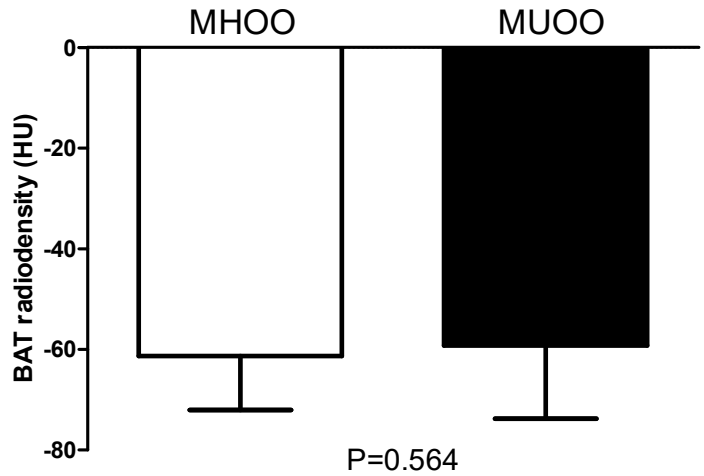

Figure 48. Mean brown adipose tissue (BAT) radiodensity in metabolically healthy overweight-obese (MHOO) (n=33) and metabolically unhealthy overweight-obese (MUOO) (n=19) participants. Dara are adjusted means and standard error. P from analysis of covariance adjusting by sex. HU: Hounsfield units.

Skeletal muscle glucose uptake after a standardized cold stimulation was higher in MHOO than in MUOO, yet it did not reach statistical significance (1.15, 1.20, 1.21, 1.22-fold for all, deep, cervical and cold sensitive muscles respectively, all P<0.1). Similar results were observed when adjusting by BMI. When adjusting by HOMA, all and deep skeletal muscle glucose uptake was significantly higher in MHOO (both P<0.033), whereas cervical muscles (P=0.062), and cold sensitive muscles (P=0.173) differences remained the same.

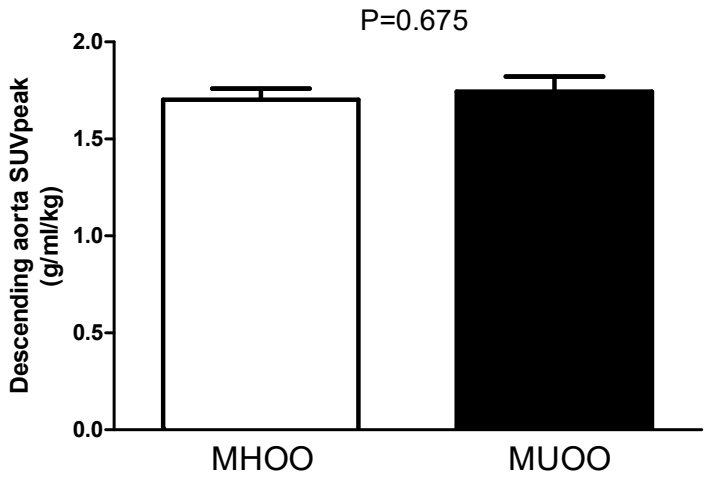

Figure 49. Descending aorta 18F-Fluorodeoxyglucose uptake in metabolically healthy overweight-obese (MHOO) (n=34) and metabolically unhealthy overweight-obese (MUOO) (n=19) participants. Dara are adjusted means and standard error. P from analysis of covariance after adjusting by sex. SUV: Standardized uptake value.

Descending aorta glucose uptake after a standardized cold stimulation was similar between groups (P=0.564, Figure 49). Similarly, there were no between group differences in glucose uptake after a standardized cold stimulation in the tricipital subcutaneous WAT (P=0.584; data not shown).

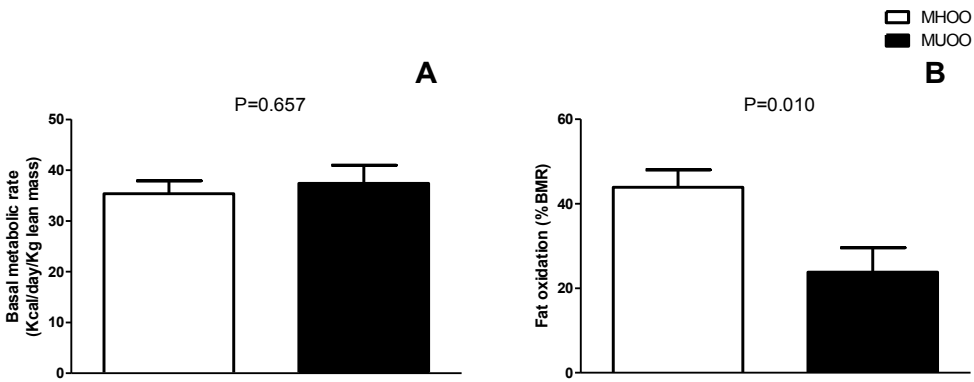

Figure 50. Basal metabolic rate (BMR) and basal fat oxidation percentage in metabolically healthy overweight-obese (MHOO) (n=26) and metabolically unhealthy overweight-obese (MUOO) (n=14) participants. Data are adjusted means and standard error. P from analysis of covariance after adjusting by sex.

BMR was similar in MHOO and MUOO (P=0.657, Figure 50A), whereas the percentage of BMR being produced by FATox was significantly higher in MHOO (1.84-fold higher; P=0.010). These results remained after adjusting by BMI or HOMA (data not shown). MFO (either expressed in absolute values or relative to lean mass) and Fatmax was similar in MHOO and MUOO (both P>0.73; Figure 51).

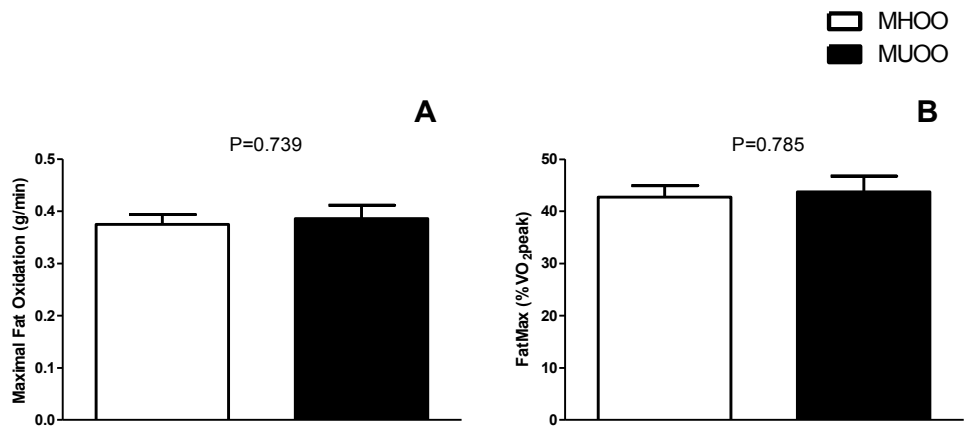

Figure 51. Maximal fat oxidation during exercise, and intensity in which maximal fat oxidation was reached (Fat Max) in metabolically healthy overweight-obese (MHOO) (n=29) and metabolically unhealthy overweight-obese (MUOO) (n=16) participants. Data are adjusted means and standard error. P from analysis of covariance after adjusting by sex. VO2peak: Maximum oxygen consumption.

Both MIT and CIT were higher in MHOO (MIT: 2.96-fold, P=0.012, Figure 52B; CIT: 3.80-fold, P=0.014, Figure 52E). There was a Time*Group interaction effect in EE induced by cold (P=0.020), and a marginal interaction effect Time*Group in energy EE by meal ingestion (P=0.092). We also observed a marginal interaction effect Time*Group in RQ values after meal ingestion (P=0.065), with both groups presenting a decreased in RQ immediately after the meal, which was rapidly reversed in MHOO. We also observed significant interaction effect Time*Group in carbohydrates and FATox after the meal

ingestion (both P<0.051; Figure 53). MHOO showed higher increases of FATox immediately after the meal, and higher CHOox levels during the rest of the record.

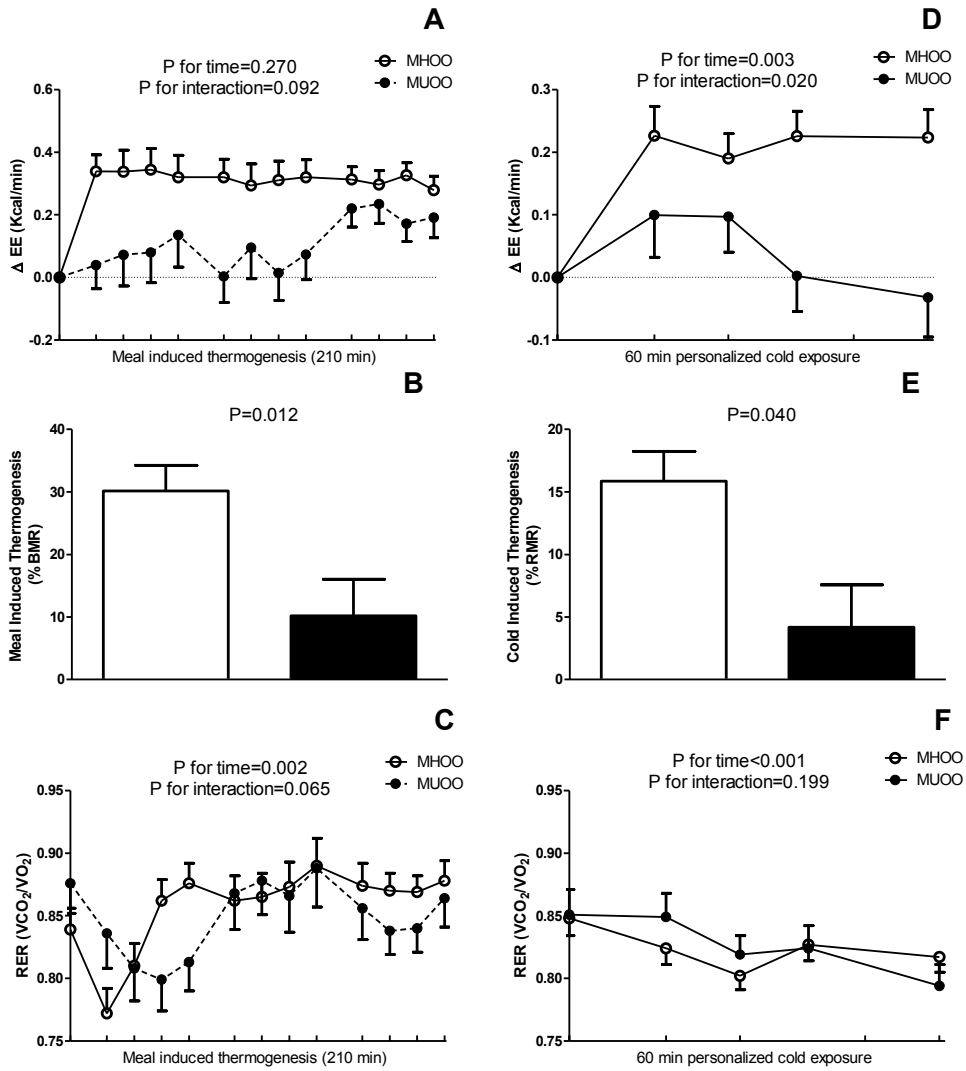

Figure 52. Adaptive thermogenesis in metabolically healthy overweight-obese (MHOO) (n=16 in panels A, B and C, and n=12 in panels D, E, F) and metabolically unhealthy overweight-obese (MUOO) (n=8 in panels A, B and C, and n=6 in panels D, E, F) individuals. A and C: P for main effect and interaction (Time*Group effect, analysis of covariance after adjusting by sex) correction of Huynh-Feldt. B and E: P values for analysis of covariance after adjusting by sex (results remained significant in B (p=0.004) and were attenuated in E (P=0.143) when adjusting by body mass index). D and F: P for main effect and interaction (Time*Group effect, analysis of covariance adjusting by sex) assuming sphericity. EE: Energy expenditure; BMR: Basal metabolic rate; RMR: Resting metabolic rate; RER: Respiratory exchange ratio; VO2: Oxygen consumption; VCO2: carbon dioxide production.

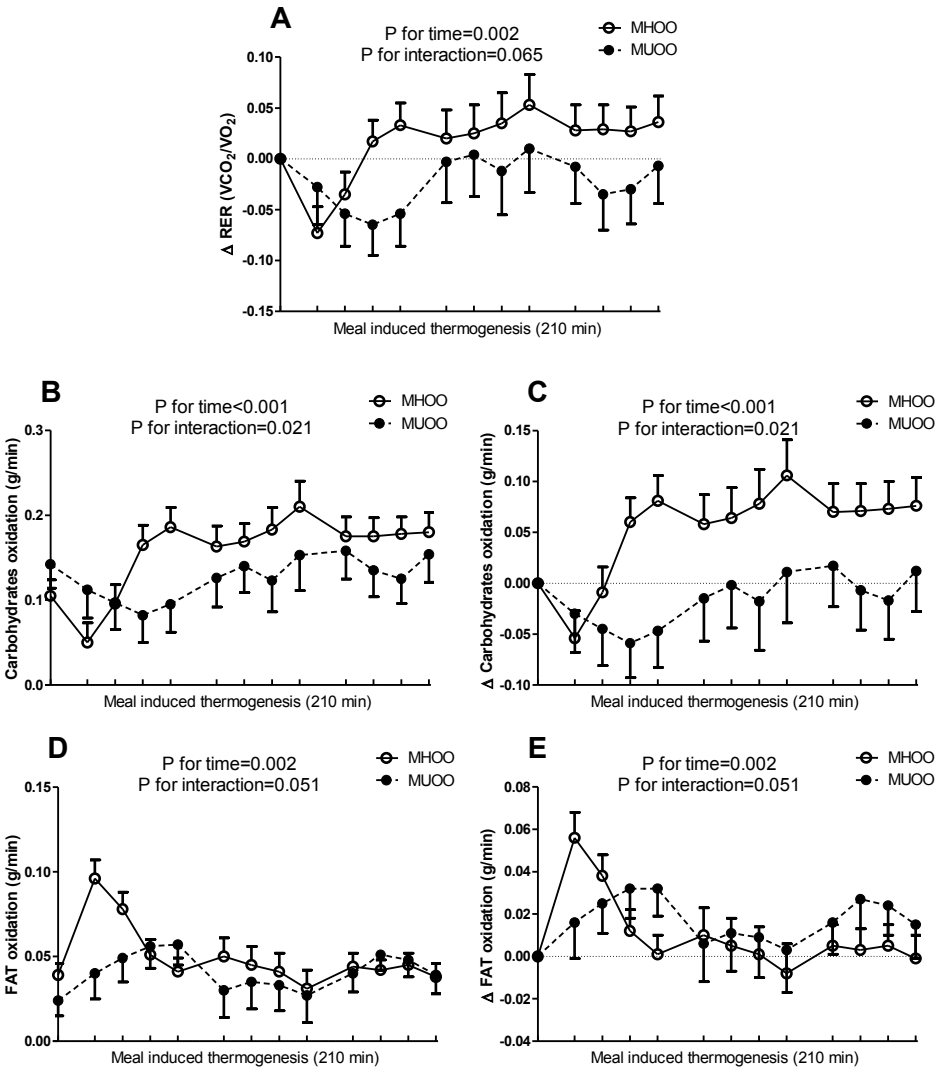

Figure 53. Metabolic flexibility in response to a standardized mixed meal in metabolically healthy overweight-obese (MHOO) (n=16) and metabolically unhealthy overweight-obese (MUOO) (n=8) individuals. Panel A: Delta in respiratory exchange ratio (RER) from basal levels. Panel B and C: Carbohydrate oxidation in response to the mixed meal; Panels D and E: Fat oxidation in response to the mixed meal. P for main effect and interaction (Time*Group effect, analysis of covariance after adjusting by sex) correction of Huynh-Feldt.

Energy intake, both measured with the ad libitum meal and estimated through the 24 h recalls, was not different in MHOO than in MUOO (Both P>0.22, Figure 54 A and B). Moreover, we observed similar patterns for hunger sensations in both groups (P=0.623, Figure 54C). On the other hand, fullness sensation was higher in the MHOO after the standardized breakfast and similar during the rest of the day, although the interaction effect did not reached significance (P=0.199, Figure 54D).

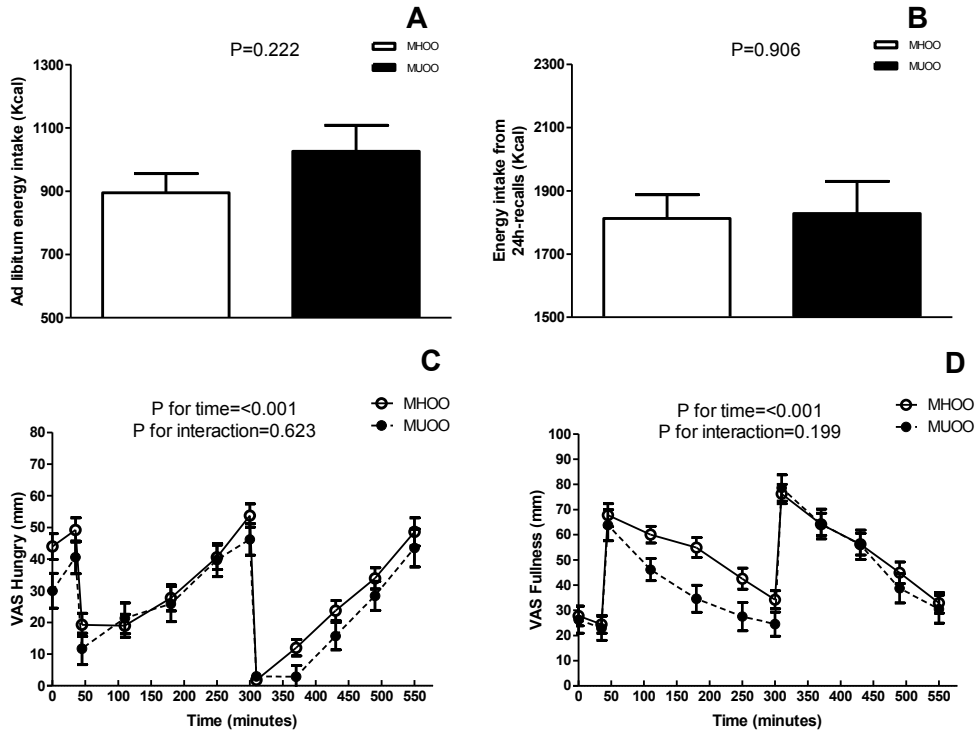

Figure 54. Energy intake and appetite-related sensations in metabolically healthy overweight-obese (MHOO, n=32 in panel A, n=33 in panel B, and n=31 in panels C and D) and in metabolically unhealthy overweight-obese (MUOO, n=18 in panel A, n=19 in panel B, and n=18 in panels C and D). A and B: P values for analysis of covariance after adjusting by sex. C and D: P for main effect and interaction (Time*Group effect, analysis of covariance adjusting by sex) assuming sphericity.

Figure 55 shows mean water temperature of the cooling vest during the personalized cold exposure before the PET-CT. We found a trend to lower water temperature in the MUOO than in the MHOO group (P=0.077).

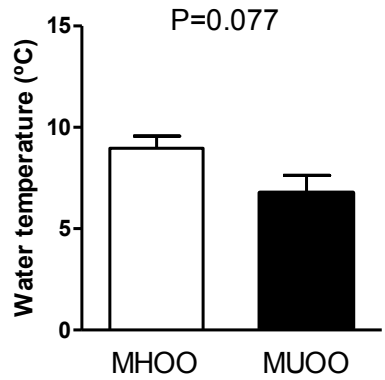

Figure 55. Cooling vest water temperature during the personalized cooling protocol before the 18F-FDG-PET/CT scan in metabolically healthy overweight-obese (MHOO, n=37) and in metabolically unhealthy overweight-obese (MUOO, n=19). Data are adjusted means and standard error. P from analysis of covariance after adjusting for sex.

DISCUSSION

Our data indicate that MHOO adults present higher BAT volume and activity (assessed by ^{18}F-FDG activity after a cold exposure) than MUOO. BAT differences were independent of weight status and insulin resistance. Despite BAT´s direct contribution to human EE and nutrient clearance seems negligible in humans [14–17,32], we observed that the higher BAT volume and glucose uptake observed in MHOO concurred with higher MIT, CIT, and metabolic flexibility in basal and post-prandial state. We cannot know whether both findings are independent or related phenomena, yet our results strongly suggest that BAT recruitment and activation, whole-body adaptive thermogenesis and metabolic flexibility are intrinsic characteristic of the intriguing MHOO phenotype and could therefore play a crucial role in the development of metabolic abnormalities.

MHOO individuals have higher BAT volume and glucose uptake

During the last decade, BAT has been regarded as a promising target for obesity and related comorbidities therapies [33]. Currently, ^{18}F-FDG PET-CT after a personalized cold stimulation is considered the gold-standard for human BAT in vivo quantification [21,34], despite BAT glucose uptake does not seem to importantly contribute to the brown adipocyte EE, and it is not necessarily linked to oxidative metabolism [35–37]. Moreover, it is plausible that the insulin resistance state bias BAT estimation when using ^{18}F-FDG PET-CT [37,38]. In fact, BAT ^{18}F-FDG uptake is much lower in diabetic individuals than in non-diabetic counterparts, despite similar fatty acids uptake and oxidative metabolism [39]. In our study, MHOO had lower insulin resistance than MUOO individuals, and it could be expected to find higher BAT ^{18}F-FDG uptake in MHOO, even if real differences in metabolic activity would not exist. Of note is however that BAT differences between groups remained after statistically taking into account insulin resistance or weight status. In addition, we found no differences in the reference tissue glucose uptake. Of note is also that we used a SUV threshold adjusted to body composition, as recommended by an international consensus group [34], which prevents the results to be biased by differences in body composition. Moreover, the higher BAT volume and ^{18}F-FDG activity cannot be attributed to a stronger cold stimulation in MHOO´s individuals, since the water temperature during the cooling protocol was higher in MHOO than in MUOO. Taken together, our results strongly suggest that MHOO individuals have higher BAT volume and glucose uptake, which may confirm the hypothesis of BAT recruitment and activation has a key factor in the prevention of metabolic abnormalities development in overweight or obese individuals.

It should be noted that mean BAT volume in MHOO (97.1 ± 11.2 ml) is not only higher than in MUOO (60.9 ± 18.4 ml) but even higher than the mean BAT volume of the whole ACTIBATE study cohort, including normal weight participants (n=135, 66.7% women, mean BAT volume: 70.5 ± 58.7 ml) [40]. The higher BAT volume and glucose uptake in MHOO could have two different physiological meanings. Firstly, it could be that obesity drives an increase in BAT volume to increase adaptive thermogenesis in order to counteract the positive energy balance, as firstly proposed by Rothwell and Stock in 1979 [41]. This is in agreement with the higher MIT and CIT observed in MHOO. However, it does not concur with recent data showing very minor contribution of BAT to whole-body EE in humans [14–17,42], and more importantly is in contrast to our data showing similar BAT radiodensity in MHOO and MUOO individuals. BAT radiodensity reflects fat content (although it is also

influenced by hyperaemia), and fat content is associated with brown adipocyte thermogenic capacity (i.e. the higher the radiodensity, the lower the fat content, and the higher the thermogenic capacity) [43]. Therefore, having higher BAT volume and glucose uptake but similar radiodensity, suggest that the BAT of the MHOO group is not more thermogenic per unit of volume than the BAT of the MUOO group. However, it should be noted that even having the same BAT thermogenic capacity for unit of tissue volume (inferred from the radiodensity), it still would translate to much higher BAT-related thermogenesis considering that MHOO had >2-fold higher BAT volume than MUOO. Secondly, there could be between-group differences in adipose tissue expandability, which would concur with the results on BAT radiodensity. WAT expandability is largely variable across individuals and has been suggested to play a central role in the development of the metabolic syndrome in the context of obesity [44]. According to this theory, individuals with high WAT expandability would be able to cope with nutrient excess without large ectopic fat deposition, therefore preventing the metabolic abnormalities caused by such ectopic fat accumulation (i.e. lipotoxicity). Thus, it is plausible that MHOO have higher WAT expandability. Indeed, MHOO have lower visceral adipose tissue volume [Mean difference (adjusting by sex): 80.66±45.75 g]. It is therefore biologically plausible to consider both WAT and BAT expandability as parallel phenomena, which may explain why MHOO have higher BAT volume despite having similar BAT thermogenic capacity (based on similar radiodensity levels). In this sense, having higher BAT volume could be a consequence of being MHOO, rather than a cause. Nonetheless, further studies are needed to empirically confirm these hypotheses.

Higher BAT volume might also be translated into a higher endocrine activity, as BAT is an active endocrine tissue [19]. Importantly, signals secreted from BAT (e.g. fibroblast growth factor) could importantly impact on whole-body homeostasis by providing an insulin sensitizing (and blood lipids lowering) stimulus [19]. Therefore, it is tempting to speculate with the possibility of BAT recruitment failure being a risk factor for the development of metabolic abnormalities. Further studies are warranted to confirm this intriguing hypothesis, since this may point to BAT transplantation as a possible therapy in humans, as it has been shown in animal models [45–47].

MHOO individuals have higher adaptive thermogenesis

The observed between groups differences in adaptive thermogenesis (i.e. MIT and CIT) could be partly the cause of presenting the MHOO phenotype, since individuals with higher adaptive thermogenesis could better counteract the negative effects of a positive energy balance [28]. For instance, higher MIT, at the same proportion of nutrient oxidation rates, would translate into better nutrient tampon capacity, reducing the time during which tissues are exposed to hyperglycaemia and hyperlipidaemia in the post-prandial state.

Another relevant issue, as mentioned above, is which tissues are responsible for the higher adaptive thermogenesis observed in MHOO individuals. BAT is not likely contributing enough to whole-body EE, at least in a direct manner, to explain such a large difference in adaptive thermogenesis [14–17,42]. However, our results may support the hypothesis of BAT having a relevant effect on adaptive thermogenesis through an endocrine mechanism (i.e. BAT secreted signal would activate muscle non-shivering thermogenesis) [14,18,48]. A higher muscle thermogenesis might also partially explain the

higher adaptive thermogenesis in MHOO [49]. MHOO had slightly higher skeletal muscle glucose uptake after a personalized cold exposure. Moreover, it should be noted that not having data on the skeletal muscle glucose uptake in warm ambient underestimates the cold-induced muscle glucose uptake in MHOO. Glucose uptake in muscle is closely related to glucose oxidation [27]. Therefore, the higher resting FATox in MHOO probably translates to lower basal muscle glucose uptake in warm environment. Thus, if MHOO have lower basal muscle glucose uptake levels the observed differences after a personalized cold exposure would represent a much a larger difference in cold-induced skeletal muscle glucose uptake.

Interestingly, we observed that the higher MIT in MHOO concurred with a higher rating for perceived fullness during the post-prandial period (Figure 54). MIT and satiety has previously been linked in humans [50]. Although our data does not allow to infer any causality, it is tempting to speculate with this increased post-prandial fulness sensation being protective with later overfeeding, which would concur with the difference observed in the ad libitum meal energy consumption (895±60 kcal vs 1025±82 kcal for sex-adjusted mean estimation in MHOO and MUOO, respectively, P=0.222).

MHOO individuals are more metabolically flexible

We observed higher resting FATox in MHOO, which is considered a sign of higher metabolic flexibility to periodic fasting [26,27], since it helps to prevent glycogen depletion in a moment in which energy requirements can be covered by FATox. Moreover, we observed a Group x Time interaction in the analyses of RQ changes after the meal ingestion. These differences are reflected in a higher FATox in the first phase of the post-prandial period in the MHOO group, and a higher carbohydrate oxidation after the first 35 minutes of the post-prandial period (Figure 53). We provided the participants with a mixed meal, which is expected to produce an increase in the RQ and carbohydrate oxidation from the beginning of the post-prandial period [26]. However, it should be noted that the liquid was at 4ºC, which might have decreased core body temperature [51,52]. Moreover, it should be noted that both CIT is highly dependent on FATox [53]. Thus, the increase in FATox in the beginning of the post-prandial period is likely due to the thermoregulation system function than to the digestion, absorption, transport and storage of nutrients. Taken together, our results suggest that the MHOO group present higher metabolic flexibility in response to both a decrease in core body temperature and the ingestion of a mixed meal.

In contrast to the higher metabolic flexibility observed in the basal and post-prandial states in the MHOO group, we did not observe any differences on metabolic flexibility during exercise (i.e. MFO) or during the cold exposure. Paradoxically, the above described higher metabolic flexibility in response to a cold liquid ingestion seems to be in contrast with the lack of a difference in the RQ during the cold exposure. However, it should be noted that the kind of cooling protocol we applied is not likely to change core body temperature [54], and thus, it seems plausible that the ingestion of a cold liquid elicit a much higher response, therefore allowing to appreciate the real differences in metabolic flexibility between groups. Alternatively, it could mean that the increase in FATox in the post-prandial state is not just a consequence of the cold ingestion, but of a combination between the meal intake (MIT) and the thermoregulatory system (CIT).

Some limitations should be considered when interpreting the results of the present study. First, the observational design of the study does not allow to infer any causal relationship. Second, it should be noted that although ^{18}F-FDG PET-CT is currently considered the gold-standard for BAT in vivo quantification, it does not allow to quantify BAT thermogenesis. Moreover, using a glucose analogue to compare two groups with different insulin sensitivity could bias the results. However, we observed that adjusting the analyses by HOMA did not modify the results. Nevertheless, using ^{18}F-FDG PET-CT clearly limits the assessment of muscle metabolism. Third, due to the low sample size, we pooled together overweight and obese participants, rather than analysing only obese. When including only obese participants (n=21; 10 MHO and 11 MUO), similar tendencies were observed for BAT volume and glucose uptake (1.75, 1.74, 1.33, and 1.70-fold higher for BAT volume, metabolic activity, SUVmean, and SUVpeak, all P>0.197), as well as for resting EE and fuel oxidation (data not shown). Unfortunately, the low sample size available for some analyses (i.e. MIT and CIT) does not allow to exclude overweight participants from the analyses and precludes to make further adjustments.

This study has important clinical implications. Our results indicate that BAT volume and activity as well as whole-body adaptive thermogenesis and metabolic flexibility are crucial characteristic of the intriguing MHOO phenotype. Therapies able to modify these features could be potentially effective in the prevention of metabolic abnormalities and cardiovascular disease development in the context of obesity.

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SECTION 4

EFFECT OF EXERCISE ON BROWN ADIPOSE TISSUE AND ENERGY BALANCE REGULATION

Effects of an exercise training program on brown adipose tissue, energy balance and metabolism in young adults:
preliminary findings from the ACTIBATE study

STUDY 10

BACKGROUND

Physical exercise exert important beneficial effects on human health [1] trough mechanism still partially unknown [2]. BAT activation and WAT browning has been recently proposed as one of the mechanisms by which exercise induces health benefits [3–6]. In murine models, it has been consistently shown that exercise induce browning of WAT depots [5–8]. In humans exercise up-regulates several endocrine mechanisms that seem to induced WAT browning [4,9–11]. In contrast, exercise also induces the up-regulation of some pathways able to inhibit BAT activity and reduce WAT browning [12–15]. Exercise is an energy demanding and heat producing process, and therefore, it would be expected that BAT, a specialized tissue for heat production, would be down-regulated in response to exercise. Three independent research groups have observed lower BAT levels in endurance trained individuals than in sedentary counterparts [16–18], although the observational design and some studies' limitations [19] prevent from drawing definitive conclusions.

To or knowledge, 3 studies has investigated the effect of exercise on human BAT metabolism [20-21], and only one [20] assessed BAT with a ^{18}F-FDG PET-CT. They showed that BAT insulin-stimulated ^{18}F-FDG activity was reduced after a 6 weeks exercise training program (i.e. combining high-intensity interval training and moderate-intensity training) in participants with high BAT volume at baseline [20]. This is partially in contrast with another study showing increased UCP-1 expression in subcutaneous WAT after an exercise program [22]. Importantly, the lack of control groups in these studies precludes from drawing definitive conclusions on the effect of exercise on human BAT. Finally, Tsiloulis et al. did not observe any change in browning markers expression after 6 weeks of training in a group of 6 participants [21]. In summary, available evidence is inconclusive, and further studies are urgently needed to determine the effect of exercise on human BAT metabolism, and whether those changes are associated to changes in energy balance regulation.

This study aimed to study the effect of a 6 months exercise training program on BAT and skeletal muscle ^{18}F-FDG activity. In addition to BAT and skeletal muscle ^{18}F-FDG activity, we measured BMR and nutrient oxidation, MIT and nutrient oxidation, CIT, energy intake, and body composition before and after the intervention.

METHODS OVERVIEW

General overview

A total of 82 participants (57 women) completed the baseline and post-intervention evaluations for at least one of the variables included in this study (Figure 2). As stated above, participants were randomly allocated to any of the three groups (Table 35).

Table 36 shows the methodology overview. For a more detailed methods description see "Methods" section.

Table 35. Participants descriptive characteristics.

	All (n=82)		Control group (n=28)		Mod-int group (n=27)		Vig-int group (n=27)		P
	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)	
Sex (n women. %)	57	(59.5)	17	(60.7)	20	(74.1)	20	(74.1)	0.460
Age (years-old)	22.09	(2.23)	22.21	(2.09)	21.80	(2.18)	22.25	(2.46)	0.716
BMI (Kg/m^2)	24.83	(4.52)	24.33	(4.59)	25.09	(4.48)	25.10	(4.61)	0.776
LMI (Kg/m^2)	14.64	(2.41)	14.79	(2.67)	14.60	(2.04)	14.53	(2.55)	0.922
FMI (Kg/m^2)	8.71	(2.89)	8.24	(2.78)	8.94	(3.08)	8.95	(2.86)	0.609
Fat mass (%)	35.40	(7.23)	34.05	(6.79)	36.00	(8.11)	36.20	(6.82)	0.508
Visceral adipose tissue (g)	337.9	(182.2)	320.5	(185.3)	343.3	(170.9)	350.5	(195.6)	0.832
Δ Ambient temperature (ºC)	1.21	(6.5)	1.33	(6.7)	1.64	(6.2)	0.68	(6.8)	0.871
Day of the year in the first evaluation (natural day)	305.3	(17.8)	305.6	(17.5)	305.7	(17.7)	304.5	(19.0)	0.965

Data are mean and standard deviation except otherwise stated. P value from a one-way analysis of variance (ANOVA), except in sex, where P value is from a Chi-square test. Δ Ambient temperature is the difference between the mean outdoor temperature for the 7 days previous to the brown adipose tissue assessment in the two evaluations (Post-Pre). BMI: Body mass index; LMI: Lean mass index; FMI: Fat mass index.

Statistical analyses

Only participants having attended >70% of training sessions were included in the analyses. Moreover, for main analyses we selected participants with an adherence to the prescribed time at the target training intensity >70% for the moderate-intensity group and >40% for the vigorous-intensity group.

We used two different statistical analyses to test the effect of the exercise intervention: a) Two-factor (Group*Time) ANOVA and; b) One-factor ANOVA comparing the exercise changes over time (after intervention minus baseline), adjusting by the baseline values. For BAT and skeletal muscle ^{18}F-FDG activity related variables we conducted additional analyses adjusting by the date when the first PET-CT was performed, and the difference between the water temperature in the cooling protocol before the PET-CT in the two evaluations. We conducted LSD-Tuckey post-hoc comparisons.

Descriptive statistics are presented as mean ± SD, unless otherwise stated. The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 21.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set at <0.05.

Table 36. Study 10 methodology.

GENERAL INFORMATION	
General aim	To study the effect of the exercise program on BAT and skeletal muscle ^{18}F-FDG activity and energy balance related variables
Design	Randomized controlled trial
Cohort and participants	ACTIBATE (n=82)
OUTCOMES INCLUDED IN THE STUDY	
Outcome	Variables included
PET-CT	BAT volume (-190/-10 HU; Ind. SUV threshold) BAT metabolic activity (-190/-10 HU; Ind. SUV threshold) BAT SUVmean (-190/-10 HU; Ind. SUV threshold) BAT SUVpeak (-190/-10 HU; Ind. SUV threshold) All muscles SUVpeak Deep muscles SUVpeak Cervical muscles SUVpeak Cold sensitive muscles SUVpeak Tricipital subcutaneous WAT SUVpeak Descending aorta SUVpeak
BIC	BMR BMR/lean mass Basal FATox
PPIC	MIT Maximum meal-induced CHOox change (i.e. delta) Maximum meal-induced FATox change (i.e. delta)
CEIC	CIT
APP	Ad libitum energy intake
BCa	Body weight BMI Lean mass Fat mass Fat mass percentage Visceral adipose tissue mass

BAT: Brown adipose tissue; ^{18}F-FDG: ^{18}F-Fluorodeoxyglucose; PET-CT: Positron emission tomography-Computerized tomography; Ind: Individualized; SUV: Standardized uptake value; BIC: Basal indirect calorimetry; BMR: Basal metabolic rate; FATox: Fat oxidation; PPIC: Post-prandial indirect calorimetry; MIT: Meal-induced thermogenesis; CHO: Carbohydrates oxidation; CIT: Cold-induced thermogenesis; APP: Energy intake and appetite regulation; BCa: Body composition assessment; BMI: Body mass index.

RESULTS

Table 35 show the descriptive characteristics of the study participants. There was not significant between groups differences in any of the descriptive variables (all P>0.460). We observed large intra-group variability in the delta (after-intervention minus baseline) in outdoor temperature when the PET-CT was performed (Range: -7.7/14.3 for all groups).

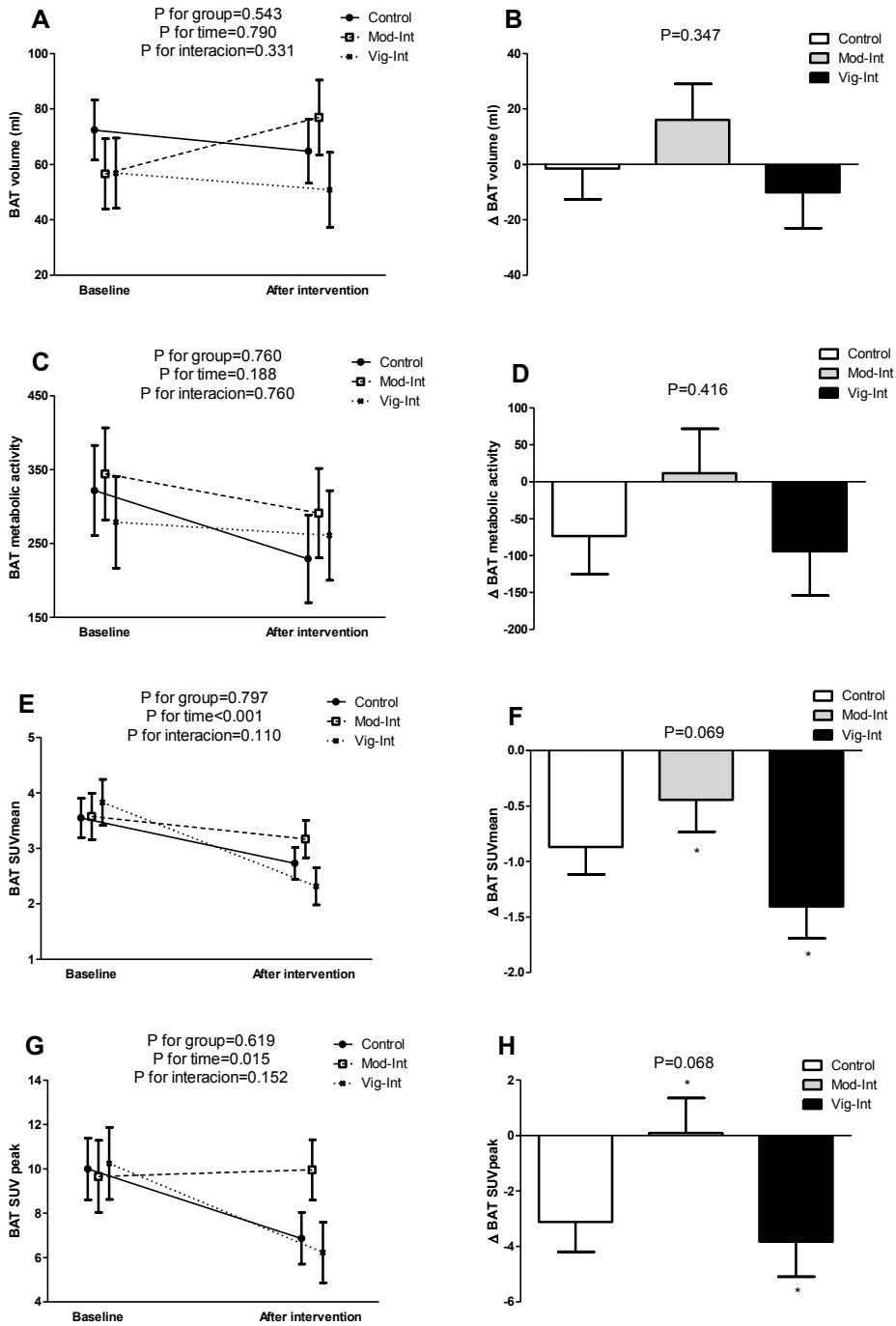

Figure 56. Effect of exercise on brown adipose tissue (BAT). Panels A, C, E and G show a two factor Time*Group analysis of variance (ANOVA). Panels B, D, F and H show a one-factor analysis of covariance (ANCOVA) comparing the differences (Post-pre) adjusted by the baseline value. Control group n=26; Moderate intensity (Mod-Int) group n=19; Vigorous intensity (Vig-Int) group n=19. Represented values are adjusted means and standard errors.SUV: Standardized uptake value. * Indicates significant differences (post-hoc comparisons).

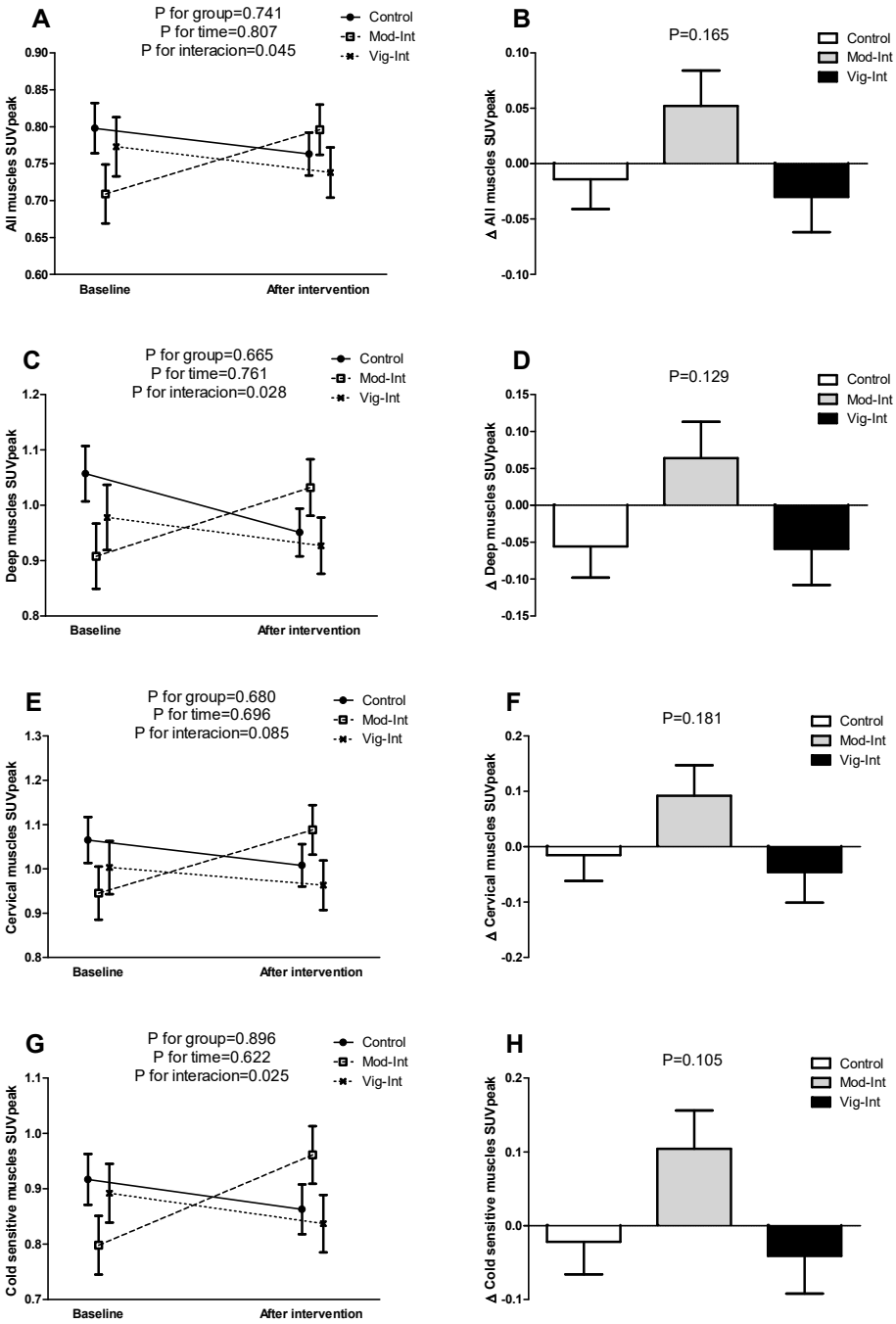

Figure 57. Effect of exercise on skeletal muscle ^{18}F-Fluorodeoxyglucose activity after a cold stimulation. Panels A, C, E and G show a two factor Time*Group analysis of variance (ANOVA). Panels B, D, F and H show a one-factor analysis of covariance (ANCOVA) comparing the differences (Post-pre) adjusted by the baseline value. Control group n=26; Moderate intensity (Mod-Int) group n=19; Vigorous intensity (Vig-Int) group n=19. Represented values are adjusted means and standard errors. SUV: Standardized uptake value.

BAT volume and metabolic activity did not change after the exercise intervention (all P>0.33; Figure 56 A-D), and results remained after adjusting by the date when the PET-CT was performed, and the difference of the water temperature used in the cooling protocol of both evaluations (data not shown). Interaction Group*Time effects were not significant for BAT SUVmean and SUVpeak (all P>0.11 Figure 56 E and G), which also remained after adjusting by cofounders (data not shown). However, marginal significance was obtained when comparing the changes in BAT SUVmean and SUVpeak adjusting by the baseline (both P<0.069; Figure 56 F and H). Post-hoc comparisons revealed that the deltas in BAT SUVmean and SUVpeak were significantly lower in the vigorous-intensity group than in the moderate-intensity group (Figure 56 F and H). These results also remained after adjusting by confounding variables (all P<0.07; data not shown).

There was a significant Group*Time interaction effect in all, deep and cold sensitive muscles (Figure 57 A, C and E), with the moderate-intensity group showing an increased SUVpeak between the baseline and after intervention values, and the control and vigorous intensity groups showing a decrease. These results persisted after adjusting by the date when the PET-CT was performed, and the difference of the water temperature used in the cooling protocol of both evaluations (data not shown). When comparing the deltas adjusting by the baseline measurement we found no significant differences (all P>0.1; Figure 57 B, D, F and H). After adjusting by the date when the PET-CT was performed and by the difference of the water temperature used in the cooling protocol of both evaluations, marginal significance was obtained for cold-sensitive muscles (P=0.083; data not shown).

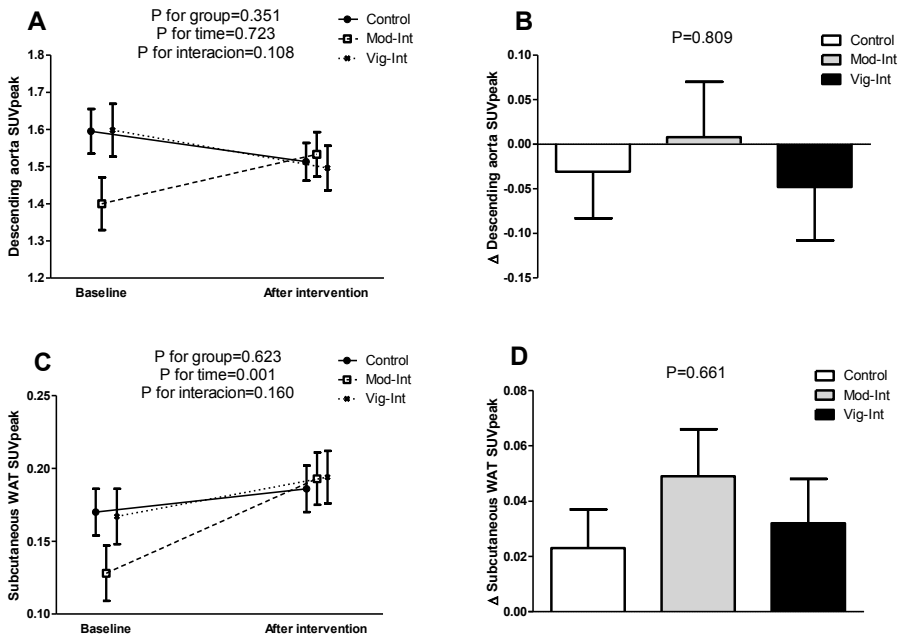

Figure 58. Effect of exercise on descending aorta and subcutaneous white adipose tissue ^{18}F-Fluorodeoxyglucose activity after a cold stimulation. Panels A, and C show a two factor Time*Group analysis of variance (ANOVA). Panels B, and D show a one-factor analysis of covariance (ANCOVA) comparing the differences (Post-pre) adjusted by the baseline value. Control group n=26; Moderate intensity (Mod-Int) group n=19; Vigorous intensity (Vig-Int) group n=19. Represented values are adjusted means and standard errors. SUV: Standardized uptake value.

The ^{18}F-FDG activity of the reference tissues (i.e. descending aorta and subcutaneous tricipital WAT) was not modified by the intervention (all P>0.1; Figure 58). These results remained when adjusting by confounders for both types of analyses. However, the Group*Time interaction effect for the descending aorta SUVpeak became significant after adjusting by the date when the PET-CT was performed and by the difference of the water temperature used in the cooling protocol of both evaluations (P=0.033; data not shown).

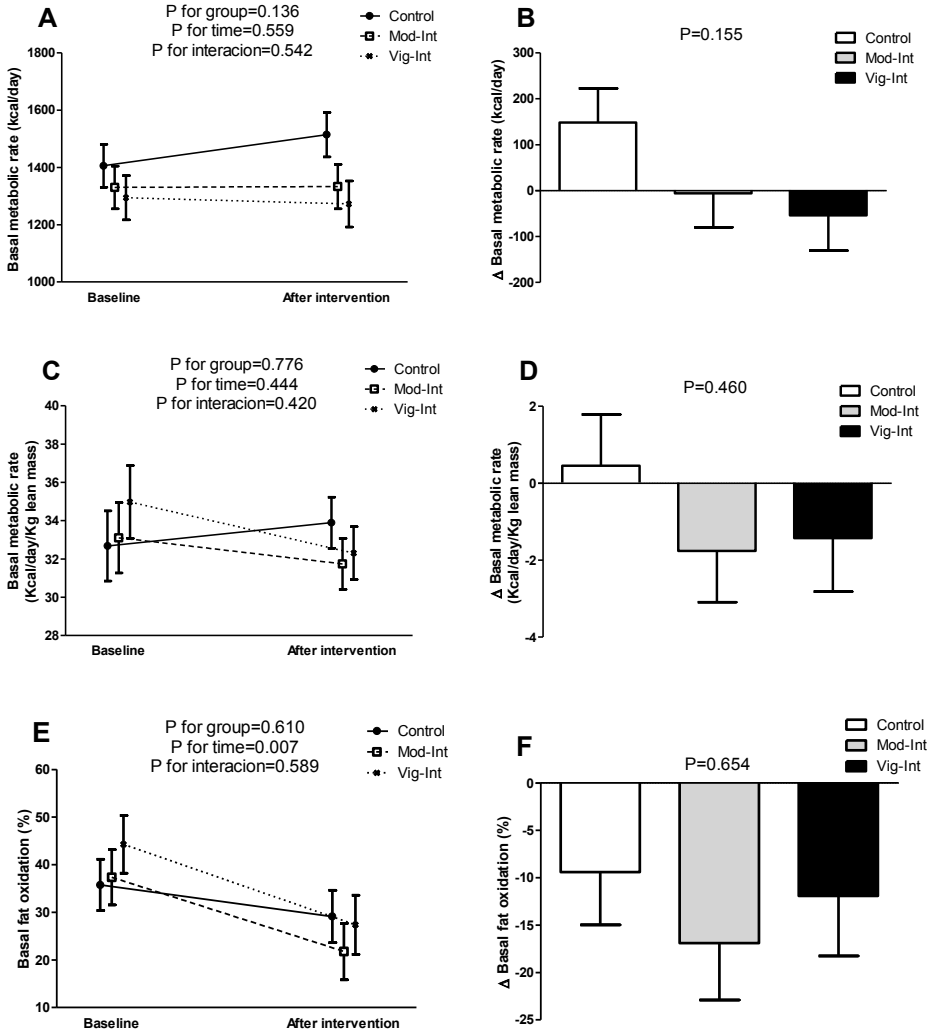

Figure 59. Effect of exercise on basal energy expenditure and nutrient oxidation rate. Panels A, C, and E show a two factor Time*Group analysis of variance (ANOVA). Panels B, D, and F show a one-factor analysis of covariance (ANCOVA) comparing the differences (Post-pre) adjusted by the baseline value. Control group n=15 (14 in fat oxidation analysis); Moderate intensity (Mod-Int) group n=15 (12 in fat oxidation analysis); Vigorous intensity (Vig-Int) group n=14 (11 in fat oxidation analysis). Represented values are adjusted means and standard errors. SUV: Standardized uptake value.

There was no change on BMR or basal FATox after the training intervention (all P>0.15; Figure 59). MIT was not modified neither (both P>0.15; Figure 60 A and B). There was a significant Group*Time interaction effect on the meal-induced CHOox analysis

(P=0.030; Figure 60C), with both exercise groups showing an increase in meal-induced CHOox in opposition to the decreased observed in the control group. There was not significant interaction effect for meal-induced FATox, although post-hoc comparisons revealed that it was significantly different in the vigorous intensity group (in which FATox was decreased) than in the control group (in which FATox was increased). There was not significant Group*Time interaction effect for CIT (P=0.682; Figure 61A). However, marginal significance was obtained when comparing the CIT deltas (P=0.076; Figure 61B), and post-hoc comparisons revealed significant differences between the control group, showing an increase, and the moderate-intensity group showing a decrease (Figure 61B). We did not observe any significant effect of the intervention on the ad libitum energy intake (both P>0.68; Figure 62).

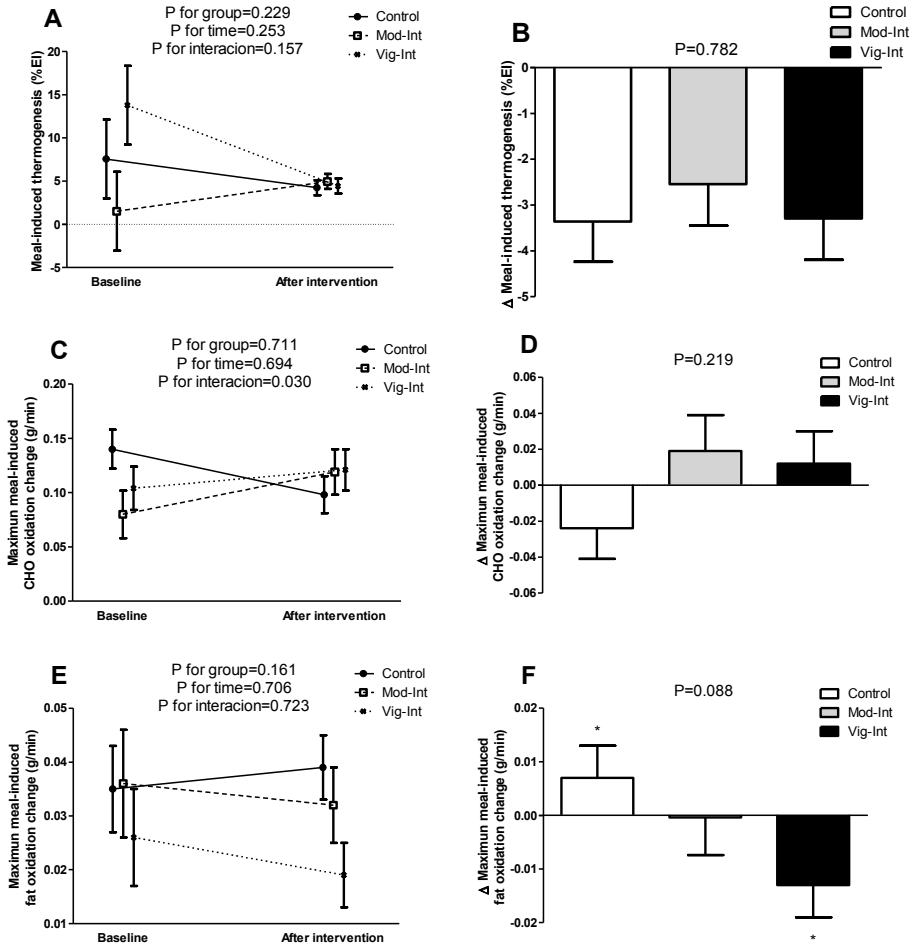

Figure 60. Effect of exercise on meal-induced energy expenditure and nutrient oxidation rates. Panels A, C, and E show a two factor Time*Group analysis of variance (ANOVA). Panels B, D, and F show a one-factor analysis of covariance (ANCOVA) comparing the differences (Post-pre) adjusted by the baseline value. Control group n=11 (12 in fat oxidation analysis); Moderate intensity (Mod-Int) group n=11 (8 in fat oxidation analysis); Vigorous intensity (Vig-Int) group n=11 (10 in fat oxidation analysis). Represented values are adjusted means and standard errors. SUV: Standardized uptake value. * Indicates significant differences (post-hoc comparisons).

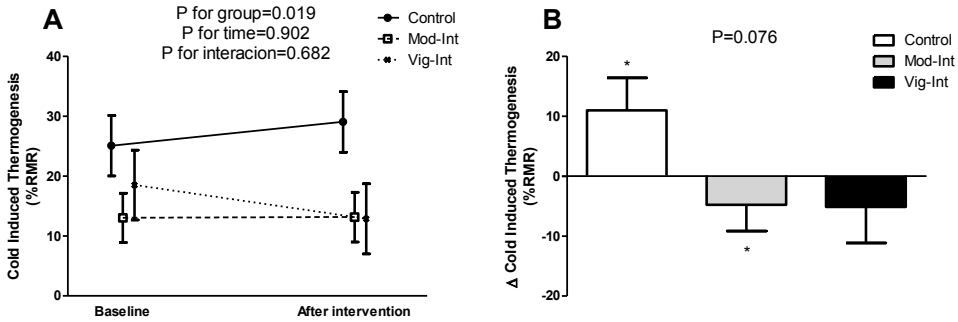

Figure 61. Effect of exercise on cold-induced energy expenditure. Panel A shows a two factor Time*Group analysis of variance (ANOVA). Panel B shows a one-factor analysis of covariance (ANCOVA) comparing the differences (Post-pre) adjusted by the baseline value. Control group n=8; Moderate intensity (Mod-Int) group n=12; Vigorous intensity (Vig-Int) group n=6. Data included in these analyses are not filtered by adherence to training intensity. Represented values are adjusted means and standard errors. SUV: Standardized uptake value. * Indicates significant differences (post-hoc comparisons).

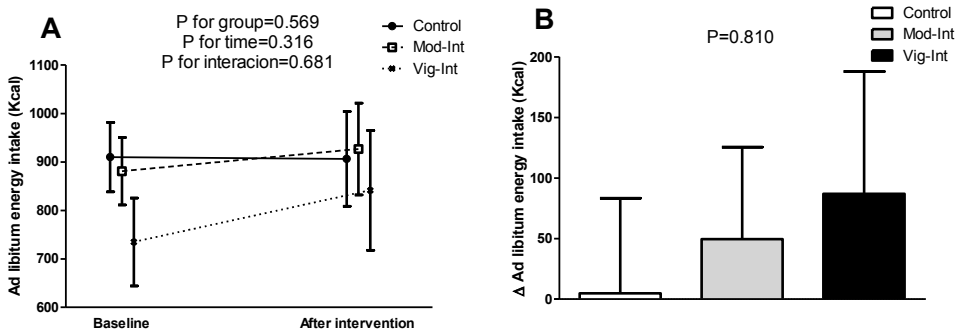

Figure 62. Effect of exercise on ad libitum energy intake. Panel A shows a two factor Time*Group analysis of variance (ANOVA). Panel B shows a one-factor analysis of covariance (ANCOVA) comparing the differences (Post-pre) adjusted by the baseline value. Control group n=16; Moderate intensity (Mod-Int) group n=17; Vigorous intensity (Vig-Int) group n=10. Represented values are adjusted means and standard errors. SUV: Standardized uptake value.

Body weight and BMI were not modified after the intervention (all P>0.12, Figure 63), although significant differences were observed in the post-hoc comparison for body weight delta between the control group and the vigorous intensity group. Lean mass was significantly increased in all groups (time effect P<0.001; Figure 64A). Moreover, significant Group*Time interaction effects were obtained for fat mass, fat mass percentage and VAT mass (all P<0.023; Figure 64 C, E and G), which was also reflected on significant differences when comparing the deltas adjusting by the baseline values (all P<0.017, Figure 64 D, F, H). Fat mass and fat mass percentage was significantly decreased in both moderate and vigorous intensity group compared to the control group (Figure 64 D and F), while VAT mass was significantly decreased in the vigorous intensity group in comparison to the control group, but not in the moderate intensity group (Figure 64H).

We ran sensitive analyses by not using an intensity adherence filter and results did not change.

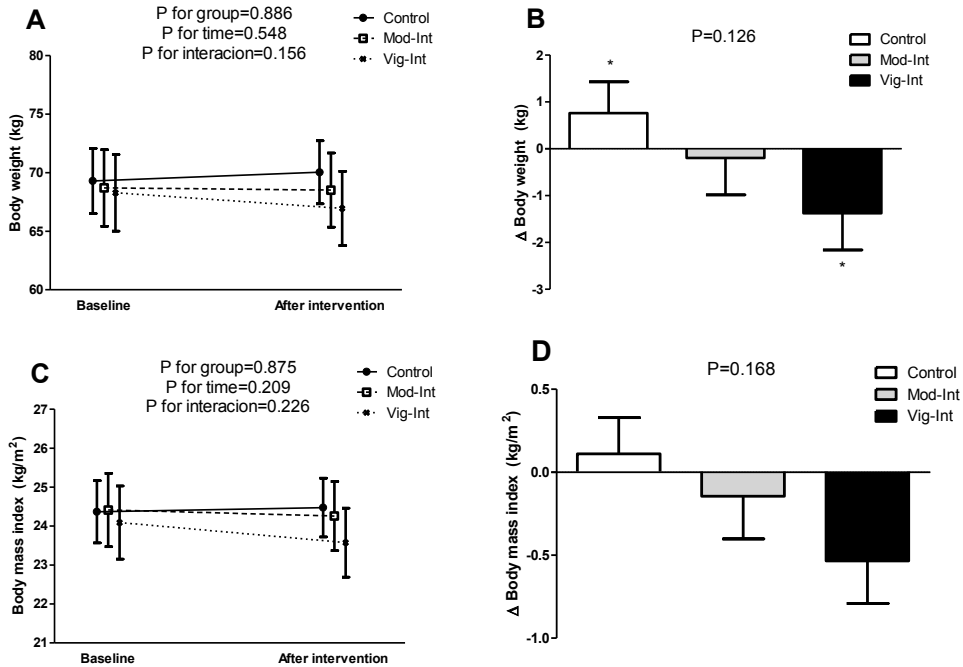

Figure 63. Effects of exercise on body weight. Panels A and C a two factor Time*Group analysis of variance (ANOVA). Panels B and D a one-factor analysis of covariance (ANCOVA) comparing the differences (Post-pre) adjusted by the baseline value. 0Control group n=29; Moderate intensity (Mod-Int) group n=21; Vigorous intensity (Vig-Int) group n=21. Represented values are adjusted means and standard errors. SUV: Standardized uptake value. * Indicates significant differences (post-hoc comparisons).

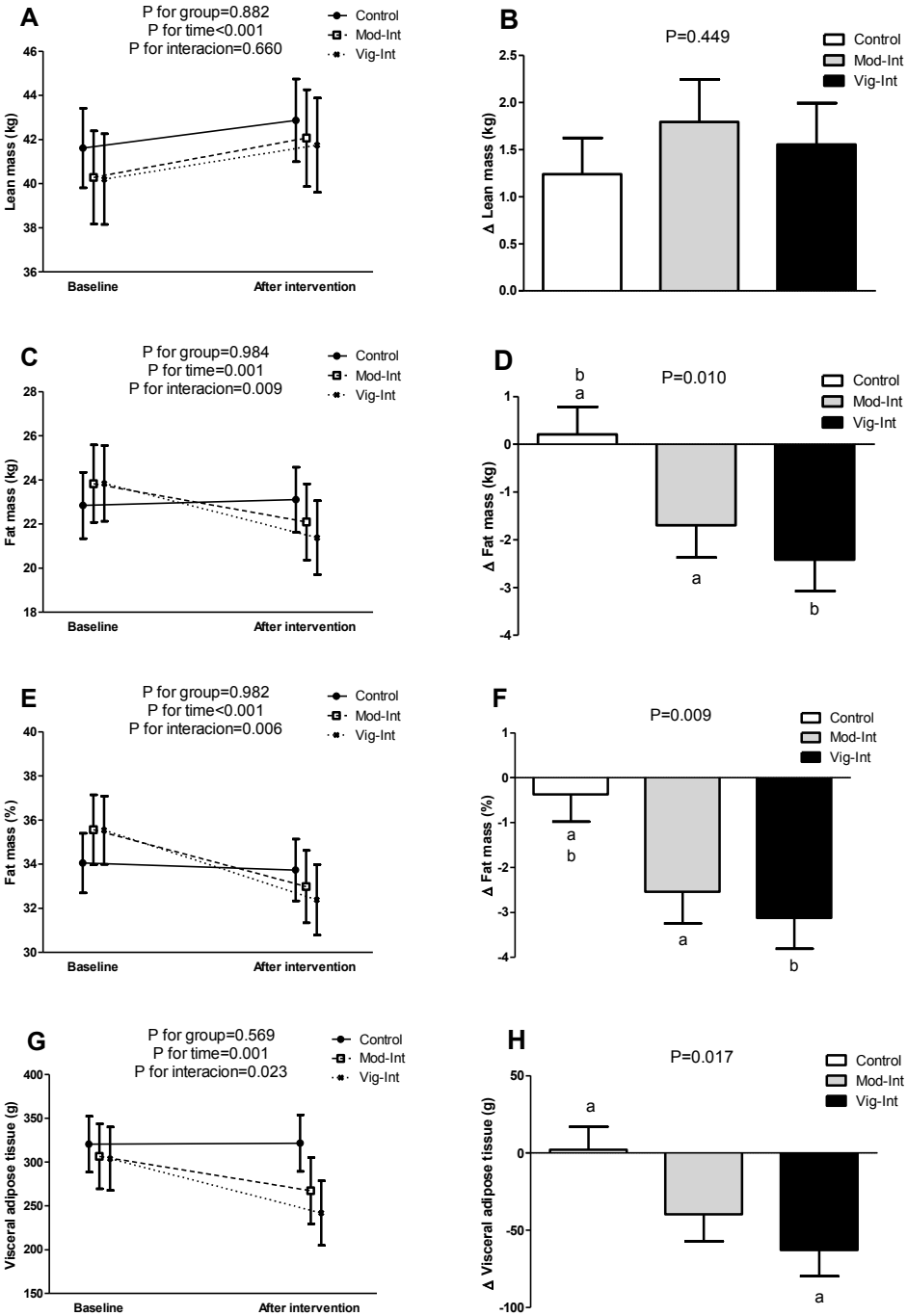

Figure 64. Effects of exercise on body composition. Panels A, C, E and G show a two factor Time*Group analysis of variance (ANOVA). Panels B, D, F and H show a one-factor analysis of covariance (ANCOVA) comparing the differences (Post-pre) adjusted by the baseline value. Control group n=26; Moderate intensity (Mod-Int) group n=19; Vigorous intensity (Vig-Int) group n=20. Represented values are adjusted means and standard errors. SUV: Standardized uptake value. Paired letters indicate significant differences (post-hoc comparisons).

DISCUSSION

A 6 months exercise-based RCT does not modify BAT volume and 18F-FDG activity after a personalized cold exposure in young adults. Moreover, basal, post-prandial and cold-induced energy expenditure, and the ad libitum energy intake did not have after the intervention. On the other hand, both exercise training groups reduced adiposity as compared with the control group. Altogether, these results show that, although the exercise program exert changes on body composition, it does not modify BAT and skeletal muscle 18F-FDG activity or energy balance related variables.

Exercise is a powerful stimulus for the prevention and treatment of many metabolic diseases [1]. Since there is biological base to believe on an exercise-induced regulation of BAT metabolism [4,6], and BAT seems to be involved in human metabolic disease development [23], it has been proposed that BAT activation and/or WAT browning could be one of the mechanisms explaining the well-proven health promoting effects of exercise [5]. However, to date, there is no conclusive evidence regarding the role of exercise on human BAT metabolism. There is only one previous study assessing BAT metabolism by PET-CT before and after an exercise intervention [20]. Motiani et al. [20] showed that insulin-stimulated BAT ^{18}F-FDG uptake was reduced after 6 weeks of exercise training in participants with high BAT volume, when merging participants having performed a high-intensity interval training program with participants having performed a moderate-intensity program [20].

In the present study, we tested the effect of an exercise program designed in agreement with the current global PA recommendations [24,25] in a randomized controlled trial design. Exercise did not significantly modify BAT volume or ^{18}F-FDG activity in young adults. These results are therefore in contrast with those of Motiani et al. [20]. However, it should be noted that Motiani et al. [20] did not include a control group, and therefore, a seasonal bias cannot be discarded. Moreover, they stimulated BAT ^{18}F-FDG uptake by insulin infusions, while we exposed participants to an individualized cold exposure, which is currently considered the method of choice for BAT in vivo quantification [26,27]. Although both insulin stimulation and cold exposure increase ^{18}F-FDG uptake, both stimulus does not induce comparable changes in blood flow regulation [28], and therefore, probably neither in BAT EE.

It should be noted however that BAT is importantly affected by seasonal changes, presenting higher volume and activity in winter than in summer [29–31]. Moreover, BAT can adapt to different temperature exposures in periods as short as 10 days [32,33]. In the present study we found a huge inter-individual variability on the difference between the ambient temperature when the PET-CT was performed in both evaluations (i.e. in seasonal variation). Therefore, seasonal variation has likely contributed to high inter-individual variability in BAT-related parameters changes, which may have blunted any exercise-induced effect. Moreover, it cannot be discarded that the training program induced WAT browning in small dispersed group of cells, which are undetectable for a PET scan due to its lack of resolution [26].

BAT SUVmean and SUVpeak were reduced in all groups from the baseline to the post-intervention measurements, which is probably explained by the larger number of participants presenting positive differences (after-intervention minus baseline) between

the outdoor temperature when both PET-CT were performed (58.4%). Nevertheless, we observed that the moderate intensity group exhibited a much lower decrease in SUVmean than the control group and the vigorous intensity group, and no decrease in SUVpeak. This suggest that the moderate intensity training could be an effective stimulus to stimulate BAT activation and/or WAT browning. Similar results were also observed for skeletal muscle SUVpeak. Both results are compatible with the hypothesis proposed by Lee et al. [34], by which muscle contractions during moderate-intensity training would mimic shivering, and therefore would increase both the muscle thermogenesis capacity and the adipose non-shivering thermogenesis (via WAT browning) by muscle secreting molecules. It is to note however that we cannot know whether the increase in skeletal muscle SUVpeak is accompanied by an increase in non-shivering [35] or shivering capacity [36]. Moreover, it does not seem plausible to assume an increase in BAT and muscle thermogenesis, since we observed a trend to a decreased whole-body non-shivering thermogenesis (Figure 62). Future studies are highly needed to examine the effect of exercise on BAT thermogenesis and on both muscle shivering and non-shivering thermogenesis.

The marginal stimulatory effect observed in the moderate-intensity group is in contrast with the inhibitory effect reported by Motiani et al. [20]. Nevertheless, it should be noted that we only found an stimulatory pattern in the moderate-intensity group, not the vigorous, and Motiani et al. [20] merged participants of both training modalities. If further confirmed, the results showing a stimulating effect of the moderate-intensity training program on BAT, seem to be in contrast with the proposed BAT role on the regulation of the redox state during exercise. If the redox pressure was the stimulus driving BAT recruitment, it would be expected that the vigorous-intensity training was more effective in stimulating BAT. Nonetheless, our results should be considered with extreme caution, since we observed a similar, but weaker, pattern in the descending aorta SUVpeak than in BAT and skeletal muscle, which suggest that other circumstances, rather than the exercise training per se are explaining the between-group differences in SUV changes.

Although an ^{18}F-FDG PET-CT after a personalized cold exposure is still considered the gold-standard for human BAT volume quantification [26,37], it presents large limitations for assessing BAT thermogenesis. Therefore, future studies applying different technologies for in vivo BAT thermogenesis assessment are needed to fully elucidate the role of exercise on human BAT metabolism. It should also be considered that changes in BAT ^{18}F-FDG uptake observed after exercise may not represent a change in BAT activity itself, but a shift to a different substrate uptake pattern, such as a more lipolytic metabolism (which is not tracked with ^{18}F-FDG-PET-CT), similarly to the effect of exercise on other tissues such as skeletal muscle [38] or even on whole-body fat utilization [39,40]. Indeed, we found an increase in post-prandial metabolic flexibility in both training groups (Figure 60C), which likely reflects this shift in energy substrate uptake patterns.

The exercise program, in both intensity modalities, induced a significant reduction of whole-body adiposity, which concurs with previous studies [1]. However, we did not observe any effect on energy balance related variables (i.e. BMR, MIT, CIT and energy intake). The effect of exercise on MIT and CIT has been very rarely studied [41]. Here we found that a combined exercise program does not influence MIT. Nonetheless, it should be noted that the training program combined stimulus that could provide different, even opposite,

results on MIT (i.e. endurance and resistance type of training) [41], and therefore future studies comparing different training types are needed before drawing firm conclusions. Regarding CIT, we observed a tendency to a decrease in both training groups when compared to the control group, which is compatible with previous studies in mice [42,43]. Nevertheless, it should be considered that the metabolic carts used present large inter-day variability [44], which may prevent us from detecting real effects.

The results of these study should be considered with caution, since there are relevant limitations. First, we presented preliminary analyses, and therefore more comprehensive analyses are needed before drawing firm conclusions. Second, following current global recommendations of PA, we combined two types of exercise (i.e. endurance and resistance) in both training groups. Since both training types are likely to produce different responses, combining then may hinder its physiological interpretation. Third, as mentioned earlier, some of the variables included in this study are largely affected by seasonality, and therefore, seasonality induced variability may blunted the exercise-induced effects. Finally, as it has been discussed, both the ^{18}F-FDG PET-CT scan and the IC measurement present relevant limitations for detecting real changes.

In summary, preliminary analyses from the ACTIBATE study shows that a combined (i.e. strength and endurance) exercise training program does not affect BAT volume and activity. However, the moderate-intensity training program could have stimulated BAT and skeletal muscle non-shivering thermogenesis capacity (increased ^{18}F-FDG activity). Future studies are highly needed to confirm these preliminary results. On the other hand, although the training program effectively reduce whole-body adiposity, we did not observe any change on BMR, MIT, CIT and energy intake.

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GENERAL DISCUSSION

ROLE OF HUMAN BROWN ADIPOSE TISSUE IN ENERGY BALANCE

The re-discovery of active BAT in human adults [1–6] raised high expectations on its potential role in energy balance regulation, being considered a promising therapeutic target against obesity and related diseases [7–9]. However, a recent study by U Din et al. [10] showed that the cold-induced EE in BAT in the cervico-upper thoracic region (15 cm long) accounted only for 4±3 kcal/day (from a mean EE of 7±5 kcal/day in warm conditions to 10±5 kcal/day in cold stress). This EE increase represent approximately a 1 % of the cold-induced increase in whole-body thermogenesis, which is in line with findings of a previous study [11]. Crucially, they also showed that the skeletal muscle (in the cervico-upper thoracic region) EE raised from 33±28 kcal/day to 86±68 kcal/day in response to non-shivering cold exposure. Importantly, all the assumptions related to BAT EE in U din et al.[10] study were based on an observed BAT mass of 133±59 grams.

It should be noted however that the currently used technology for BAT in vivo quantification presents important limitations for human BAT volume determination. Currently, ^{18}F-FDG PET-CT is considered the best available technique to determine BAT volume [12]. However, the low image resolution of the PET scan (1 cm^3) makes it uncapable of detecting small depots of beige adipocytes, which is likely the form in which most of the human thermogenic fat is presented [12]. Therefore, it has been suggested that estimations like the one made by U Din et al. [10] may have underestimated the real contribution of BAT to whole-body EE [12]. In this regards, if total BAT volume were 550 g, its total contribution to whole-body EE, upon activation, would be of 46 kcal/day [12]. Even more optimistic estimations of the total volume of inducible thermogenic adipose tissue of 2550 g [13] would translated to an EE of 211 kcal/day [12].

Besides the possible error on the estimation of total volume made in the study by U Din et al. [10], they showed that the cold-induced EE in skeletal muscle of the cervico-upper thoracic region correspond to 53 kcal/day approx., while BAT only increased EE by 4 kcal/day approx. Therefore, assuming the unreal hypotheses of that no other organs contribute to CIT [14] and that the proportion between BAT and muscle thermogenesis observed in the cervico-upper thoracic area would be applicable to whole-body (which is not feasible [13]), skeletal muscle would account for 93% of CIT, while BAT would account for 7% of CIT (which is considerably higher than the 1% estimated by cervico-upper thoracic BAT energy consumption [10,11]). In the **study 3** of this Doctoral Thesis we found that a mild cold exposure at a temperature adjusted to elicit maximum NST induces a very modest increase in EE (229.44±189.6 kcal/day). Assuming that BAT is responsible for 7% of the whole-body thermogenesis, it would translate to 16.1±13.27 kcal/day. It means that, even assuming the unlikely scenario of no contribution of other tissues to CIT, and assuming that the rate between BAT and muscle thermogenesis in the cervico-upper thoracic area applies to the rest of the body, BAT associated EE would be still rather low. Thus, our data support the notion that BAT EE is not significantly influencing whole-body EE in humans and suggest that even assuming an underestimation of total BAT volume by PET scan, BAT EE would not be enough to have a clinically meaningful impact on human energy balance.

Nonetheless, despite that recent studies strongly suggest that BAT contribution to whole-body EE is negligible, a positive association between BAT and CIT has been found by several research groups [15–18]. This, together with the fact that skeletal muscles groups located near BAT depots are the main contributors to CIT [10,14] have led to the suggestion of BAT playing a key regulatory role of human CIT through endocrine secretion [10]. If this hypothesis was true, a positive association between BAT and whole-body thermogenesis should be observed. In contrast, we did not observe any association between the BAT-related parameters and CIT in the **study 4**, which also concurs with others [14,19–21]. Similarly, we also failed to observe any association between BAT-related parameters and MIT, neither with BMR (**study 5**). The findings from the studies 4 and 5 suggest that BAT might not be orchestrating whole-body thermogenesis trough indirect mechanisms. On the other hand, **study 9** shows that MHOO young adults present both higher BAT volume and adaptive thermogenesis (i.e. CIT and MIT) than MUOO counterparts, which suggest that despite the lack of association of BAT with CIT and MIT in the whole-cohort (studies 4 and 5), BAT recruitment in response to positive energy balance (see **study 8**) may be indirectly causing a relevant increase in adaptive thermogenesis (the so-called obesity induced-thermogenesis [22]). This intriguing hypothesis is apparently in contrast with the lowest BAT radiodensity observed in participants with higher BMI (**study 8**), and the comparable BAT radiodensity observer in MHOO and MUOO (**Study 9**). Further studies are warranted to elucidate whether obesity-induced thermogenesis really exist in humans, and whether BAT expandability is orchestrating this process.

Finally, it should be considered that even if BAT significantly contributes to human whole-body EE, its potential role on energy intake regulation should be fully understood before assuming that BAT can alter human energy balance. Commonly, changes in EE (either increases or decreases) are couple with proportional changes in energy intake [23,24]. Moreover, it is biologically plausible that BAT activity influences energy intake and appetite regulation [25–27]. However, the **study 6** shows that BAT is not associated with energy intake or appetite-related sensations in young adults, which is consistent with the lack of association of BAT with CIT and MIT (**studies 4 and 5**). Moreover, the higher BAT volume in MHOO participants was not accompanied by higher energy intake (**study 9**).

In summary, although BAT is present in most human adults [28], it is highly thermogenic per unit of tissue volume [10,29], and also seems to be connected with the appetite regulation system [15], the results of the present Doctoral Thesis suggest that it is not significantly influencing human energy balance [30].

ROLE OF HUMAN BROWN ADIPOSE TISSUE AND ENERGY BALANCE REGULATION IN BODY COMPOSITION AND THE DEVELOPMENT OF THE METABOLIC SYNDROME

The lower BAT volume and ^{18}F-FDG activity observed in obese individuals in the first studies assessing human BAT by ^{18}F-FDG PET-CT [1,2,4] led to the assumption that BAT low volume or absence may predispose to weight gain in humans. However, in a systematic review included in the **study 8**, we observed that only half of the previously published

studies reported a negative association between BAT and whole-body adiposity or showed differences in BAT between obese and lean people. More importantly, almost all previous studies presented serious methodological issues that likely biased the association between BAT volume and/or activity (as assessed by ^{18}F-FDG activity) and whole-body adiposity.

In contrast to most of previous scientific literature, the **study 8** shows that BAT volume, assessed by ^{18}F-FDG PET-CT after a personalized cold-exposure and quantified strictly following the current recommendations [31], is positively associated with whole-body and central adiposity in young adults. Moreover, in **study 9**, we show that within overweight or obese participants, those preserving a healthy metabolic profile (i.e. MHOO) present much larger BAT volume and ^{18}F-FDG activity than MUOO. Altogether, the results of the present Doctoral Thesis strongly suggest that BAT hypertrophy and hyperplasia takes place as a consequence of a positive energy balance in most young individuals, and that the capacity to recruit BAT in response to weight gain might be determining the development of the metabolic syndrome. Further studies are urgently needed to empirically confirm these hypotheses. If confirmed, BAT might become a powerful therapeutic tool, not for weigh loss or preventing weight gain, but for preventing metabolic abnormalities in obese patients.

Nonetheless, until an experimental confirmation, the hypothesis of BAT expansion determining the metabolic syndrome development should be considered with caution, since it is also possible that the higher BAT is a consequence, rather than the cause, of the MHOO phenotype. For instance, mechanism governing WAT expandability, a key issue in the development of the obesity-associated metabolic problems [32], might also be governing BAT expandability. Thus, higher BAT in MHOO would be just a reflect of higher WAT expandability, which would be the determining factor in the metabolic syndrome prevention. On the other hand, even if BAT expansion takes place in parallel to WAT expansion, whether this BAT expansion determines a higher BAT endocrine activity [33] deserve to be elucidated. If confirmed, the BAT increased secreting activity might be synergistic with the WAT expansion capacity in the prevention of the metabolic syndrome.

Besides higher BAT volume and ^{18}F-FDG activity, we also found that MHOO individuals present higher adaptive thermogenesis and higher metabolic flexibility in basal and post-prandial states. However, based on previous studies, higher BAT is not likely explaining the higher adaptive thermogenesis and higher metabolic flexibility in MHOO individuals [10,14,29,30,34,35]. Therefore, it is tempting to speculate with a common mechanism explaining both the higher BAT volume and activity, and the increased thermogenesis and metabolic flexibility in MHOO.

Finally, the results of the present Doctoral Thesis also showed that BAT volume and ^{18}F-FDG activity are not associated with BMD (**study 7**). Previous studies showing positive associations between BAT related parameters and BMD in women [36,37] raised the hypothesis of BAT playing a role in the human osteogenic process [38], as it seems to be the case in murine models [38–40]. Our results suggest that the above-mentioned relationship between BAT and BMD in women might be a consequence of biased results and/or specific for a disease context. Therefore, our results suggest that BAT is not significantly influencing BMD, at least in young adults.

EFFECT OF EXERCISE ON BROWN ADIPOSE TISSUE AND ENERGY BALANCE REGULATION

Physical exercise exerts important benefits on human health, acting through mechanisms that are still partially unknown [41,42]. Since exercise stimulates both catecholamine secretion [43] and several non-sympathetic dependent BAT activators [44,45] in humans, and clearly induce WAT browning in rodents [46], it was hypothesized that WAT browning and/or BAT recruitment might be one of the mechanisms explaining the exercise-induced improvements in human health. However, in a randomized controlled trial aiming to study the effect of a 6 months combined (i.e. strength and endurance) exercise training program on BAT volume and activity, we found no clear effects of exercise on BAT-related parameters. The high variance in BAT related parameters explained by seasonality [18,47] likely contributed to lack of statistical power to detect differences. Nonetheless, we observed a trend for a positive effect of the moderate-intensity training program on BAT ^{18}F-FDG activity after an individualized cold stimulation. Therefore, future studies are highly needed to demonstrate whether moderate-intensity exercise is really able to stimulate BAT and/or induce browning in humans.

Many physiological functions have been proposed to explain the exercise-induced WAT browning. Among then, Lee et al. [48] proposed that moderate-intensity muscle contractions may mimic muscle shivering, and therefore induce a similar muscle secretome. This shivering-like muscle signaling pattern would lead to BAT recruitment and/or WAT browning, as it occurs in response to repeated cold exposure [49]. Our results showing a trend to BAT activation in response to moderate-intensity training, but not in response to vigorous-intensity training, might be considered a proof of concept of the theory proposed by Lee et al. [48]. Moreover, the moderate-intensity program also seemed to result in higher skeletal muscle ^{18}F-FDG activity after a personalized cold exposure. If confirmed, it would mean that moderate intensity training, in contrast with cold exposure, increases both adipose tissue and skeletal muscle thermogenesis capacity. Crucially, besides UCP-1 activity, others non-shivering thermogenic mechanisms are present both in BAT and skeletal muscle [50], and therefore, it is tempting to speculate with those mechanisms (such as creatine cycle or futile calcium cycle) being synergistically stimulated in both tissues.

It should be noted however, that we observed no change in any of the variables of EE (i.e. BMR, CIT, MIT) after the exercise program. Moreover, we observed a tendency to an exercise-induced decrease in CIT, which is in agreement with previous animal studies [51,52]. EE data should be therefore considered with caution since some important limitations, such as the relatively low number of participants and the relatively low inter-day reliability of the metabolic carts [53], might have prevent us from detecting existing effects.

In summary, our results show that exercise does not modify BAT volume and activity. However, we observed some tendencies that may support the notion that moderate-intensity exercise induce BAT activation/recruitment and/or WAT browning, as well as muscle thermogenesis. However, future studies are needed to fully elucidate the role of exercise on BAT metabolism. For this future studies, it should be noted that endurance and strength training may elicit different responses [54], and therefore should be tested

separately. Moreover, assessing BAT and skeletal muscle thermogenesis or oxidative metabolism, rather than a glucose analogue uptake after a cold exposure, would be also desirable.

GENERAL LIMITATIONS

The results presented in this Doctoral Thesis should be considered with caution since there are some limitations:

- Seven out of eight studies contained in the Doctoral Thesis applied observational designs, and therefore, establishing causal relationships is not possible.
- We assessed BAT by a ^{18}F-FDG PET-CT scan after a personalized cold stimulation, which is currently considered the best available technique for human BAT in vivo quantification [12,31]. However, BAT ^{18}F-FDG activity does not necessarily represent BAT thermogenesis [55], and PET image resolution might have underestimated real BAT volume [12].
- Importantly, for BAT assessment, but also for skeletal muscle ^{18}F-FDG activity and CIT quantification, we used a personalized cooling protocol based on the individuals' shivering threshold. This type of cold stimulation is currently considered adequate for this purpose, and is used by several research groups around the world [13,20,29,31,56]. However, it should be noted that we determined shivering by direct observation and by asking the participants, which is not as objective as electromyography. Moreover, muscle shivering in deep muscles is likely occurring before shivering in large superficial muscle groups (which makes it externally detectable or self-perceivable) [14]. Importantly, the ratio between deep and superficial skeletal muscle shivering might be different for different participants, and therefore, the shivering threshold method may evoke different grades of cold stimulation. Other methods for BAT and CIT assessment rather than the shivering threshold are currently under study [34,57], and thus, it would be desirable to replicate the findings of the present Doctoral Thesis applying these other methods.
- Most of the studies contained in this Doctoral Thesis included the measurement of human EE. To do so, we used two different metabolic carts which present relatively low inter-day reliability [53], which could be even more important for CIT and MIT assessment [58]. Thus, device error might have contributed to intra-individual non-biological variance.
- Many of the studies included in this Doctoral Thesis examine the association of two parameters (e.g. BAT and CIT) assessed in different days. Therefore, we cannot discard that inter-day biological variability have contributed to the lack of association.
- All the studies included in this Doctoral Thesis were carried out in a group of very young adults, mostly healthy. Therefore, both the young age of the study group and the healthy state does not allow to apply these findings to older or less healthy populations. Therefore, it is mandatory to replicate the findings of the present Doctoral Thesis on different populations.

- Regarding the longitudinal design (**study 10**), having measured the participants in different moments of the year have clearly contributed to increase the variance in the studied parameters. Therefore, although the inclusion of a control group and the homogenic distribution in the date of evaluation prevents from a non-systematic error, the seasonality associated variance is likely to have blunted any exercise-related effect on BAT metabolism.
- Despite we included two types of exercise (i.e. endurance and resistance) following the international physical activity recommendations, it is likely that endurance and resistance exercise induce different response over the study outcomes [54]. Therefore, combining them may hinder its physiological interpretation and might have prevented us from observing differential regulation on some of the studied variables.

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CONCLUSIONS

GENERAL CONCLUSION

The results of the present Doctoral Thesis show that human brown adipose tissue volume and activity are not associated with energy balance related variables. On the other hand, brown adipose tissue volume and fat content are positively associated with body adiposity, and metabolically healthy overweight or obese young adults present significantly higher brown adipose tissue volume and activity than their metabolically unhealthy counterparts. Finally, we observed that a 6 months exercise training program did not significantly modify brown adipose tissue volume and activity.

SPECIFIC CONCLUSIONS

Section 1: Role of brown adipose tissue in human energy balance.

1. A mild cold exposure at a temperature adjusted to elicit maximum non-shivering thermogenesis induces a very modest increase in energy expenditure (<40% resting metabolic rate), which is maintained constant despite a shift in nutrient oxidation rates in young adults.
2. Brown adipose tissue volume, and brown adipose tissue and skeletal muscle activity upon cold exposure are not associated with cold-induced thermogenesis and nutrient oxidation rates in young adults.
3. Brown adipose tissue volume and brown adipose tissue and skeletal muscle activity upon cold exposure are not associated with basal and post-prandial energy expenditure and nutrient oxidation rates in young adults.
4. Brown adipose tissue volume and brown adipose tissue and skeletal muscle activity upon cold exposure are not associated with energy intake and appetite related sensations in response to two different meals in young adults.

Section 2: Role of brown adipose tissue in body composition.

5. Brown adipose tissue volume and activity upon cold exposure are not associated with whole-body and lumbar spine bone mineral density in young adults.
6. Brown adipose tissue volume, but not its activity upon cold exposure is positively associated to whole-body and central adiposity, while brown adipose tissue radiodensity is negatively associated to whole-body and central adiposity in young adults.

Section 3: Role of brown adipose tissue and energy balance regulation in the development of the metabolic syndrome.

7. Brown adipose tissue volume and activity upon cold exposure, meal and cold-induced thermogenesis, and basal and post-prandial metabolic flexibility are higher in metabolically healthy overweight or obese than in metabolically unhealthy overweight or obese young adults.

Section 4: Effect of exercise on brown adipose tissue and energy balance regulation.

8. A 6 months exercise training program does not modify brown adipose tissue volume activity upon cold exposure. The exercise program reduced whole-body and central adiposity, although no changes were observed on basal metabolic rate, meal-induced thermogenesis and cold-induced thermogenesis.

ANEXES

PAPERS DERIVED FROM THE DOCTORAL THESIS

PUBLISHED/ACCEPTED PAPERS

1. **Sanchez-Delgado G**, Martinez-Tellez B, Garcia-Rivero Y, Alcantara JMA, Acosta FM, Amaro-Gahete FJ, Llamas-Elvira JM, Ruiz JR. Brown adipose tissue and skeletal muscle 18f-fdg activity after a personalized cold exposure is not associated with cold-induced thermogenesis and nutrient oxidation rates in young healthy adults. ***Front Physiol***, in press.
2. **Sanchez-Delgado G**, Alcantara JMA, Acosta FM, Martinez-Tellez B, Amaro-Gahete FJ, Ortiz-Alvarez L, Löf M, Labayen I, Ruiz JR. Estimation of non-shivering thermogenesis and cold-induced nutrient oxidation rates: Impact of method for data selection and analysis. ***Clin Nutr***, in press.
3. **Sanchez-Delgado G**, Martinez-Tellez B, Garcia-Rivero Y, Acosta FM, Alcantara JMA, Amaro-Gahete FJ, Llamas-Elvira JM, Gracia-Marco L, Ruiz JR. Association between brown adipose tissue and bone mineral density in humans. ***Int J Obes***, in press.
4. **Sanchez-Delgado G**, Alcantara JMA, Ortiz-Alvarez L, Xu H, Martinez-Tellez B, Labayen I, Ruiz JR. Reliability of resting metabolic rate measurements in young adults: Impact of methods for data analysis. ***Clin Nutr*** 2018; 37(5):1618-1624
5. **Sanchez-Delgado G**, Martinez-Tellez B, Olza J, Aguilera CM, Labayen I, Ortega FB, Chillon P, Fernandez-Reguera C, Alcantara JMA, Martinez-Avila, WD, Muñoz-Hernandez V, Acosta FM, Prados-Ruiz J, Amaro-Gahete FJ, Hidalgo-Garcia L, Rodriguez L, Ruiz, YAK, Ramirez-Navarro A, Muros-de Fuentes MA, García-Rivero Y, Sanchez-Sanchez R, de Dios Beas Jimenez J, de Teresa C, Navarrete S, Lozano R, Brea-Gomez E, Rubio-Lopez J, Ruiz MR, Cano-Nieto A, Llamas-Elvira JM, Jimenez Rios JA, Gil A, Ruiz JR. Activating brown adipose tissue through exercise (ACTIBATE) in young adults: Rationale, design and methodology. ***Contemp Clin Trials*** 2015; 45: 416–25.
6. **Sanchez-Delgado G**, Martinez-Tellez B, Olza J, Aguilera CM, Gil Angel, Ruiz JR. Role of exercise in the activation of brown adipose tissue. ***Ann Nutr Metab***. 2015; 67: 21–32.

PUBLISHED EDITORIALS AND LETTERS TO THE EDITOR

1. **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*** 2016; 35: 539–40.
2. **Sanchez-Delgado G**, Martinez-Tellez B, Gil A, Ruiz JR. Is Brown Adipose Tissue-Mediated Adaptive Thermogenesis the Missing Component of the Constrained Total Energy Expenditure Model? ***Ann Nutr Metab*** 2016; 69: 51–3.

PAPERS IN PREPARATION/SUBMITTED

1. **Sanchez-Delgado, G**. Alcantara,JMA., Acosta, FM., Martinez-Tellez, B., Amaro-Gahete, FJ., Merchan-Ramirez, E., Löf, M., Labayen, I., Ravussin, E., Ruiz, JR. Energy expenditure and macronutrient oxidation in response to an individualized non-shivering cooling protocol. ***Am J Clin Nutr.*** *Submitted*
2. **Sanchez-Delgado G,** et al. Brown adipose tissue volume and ^{18}F-FDG activity are not associated with energy intake in young human adults. *In preparation*

3. **Sanchez-Delgado G,** et al. Association of brown adipose tissue with basal and postprandial energy expenditure and nutrient oxidation rates in young adults.

4. **Sanchez-Delgado G**, et al. Unexpected evidence for a positive association between brown fat volume and body fat in young healthy adults. *In preparation*

5. **Sanchez-Delgado G**, et al. Higher brown adipose tissue, whole-body adaptive thermogenesis and metabolic flexibility in metabolic healthy overweight-obese adults than in metabolically unhealthy counterparts: a case-control study. *In preparation*

6. **Sanchez-Delgado G**, et al. Effects of exercise on human brown adipose tissue and energy balance. *In preparation*

SHORT CURRICULUM VITAE

Personally Guillermo Sánchez Delgado
Born 20th November 1989, Granada, Spain
gsanchezdelgado@ugr.es

Education

2012 Bachelor's degree in Sports Sciences (Grade: 9.1/10), University of Granada, Spain.
2013 Master's degree in Researching in Physical Activity and Sports (Grade: 9.3/10), University of Granada, Spain.
2013 Master's degree in Personal Training (Grade: 9.7/10), University of Granada, Spain.
2013-2018 PhD Student in Biomedicine, University of Granada, Spain

International internships

2015, 2018 University of Leeds, UK. Human Appetite Research Unit, School of Psychology, Faculty of Medicine and Health. Prof. John Blundell´s lab.
2018 University of Cambridge, UK. Institute of Metabolic Sciences. Prof. Antonio Vidal-Puig´s lab.

Previous Positions

2014-2018 Predoctoral FPU Research Fellow. Department of Physical Education and Sport, School of Sport Sciences, University of Granada, Granada, Spain.
2011 – 2013 Research Initiation Fellow. Department of Physical Education and Sport, School of Sport Sciences, University of Granada, Granada, Spain

Supervision

2014 – 2015 Supervisor for 4 master Thesis (Master's degree in Personal Training, University of Granada)

Research projects

2014 – 2017 ACTIBATE: Activating Brown Adipose Tissue through Exercise. Effects of an exercise intervention on activity and quantity of Brown adipose tissue: A Randomized Controlled Trial. Funded by the Spanish Ministry of Economy and competitiveness among others: ≈600000€.
2014 – 2016 ActiveBrains: Effects of an exercise randomized controlled trial on cognitive function and brain (functional and structural) in overweight and obese preadolescents. Funded by the Spanish Ministry of Economy and competitiveness among others: 120000€.
2015-2016 SPORTEUS: Effects of the intake of a protein enriched smoothie on recovery of muscular function and muscle damage after an acute bout of high intensity exercise. Funded by Lactalis-Puleva S.L.: 190000€
2018-2020 The SmartMove project: Exercise in the prevention and treatment of obesity and insulin resistance: Smart analysis-smart interventions. Funded by Spanish Ministry of Economy and competitiveness: 100000€.

Publications

1. **Sanchez-Delgado, G.**, Martinez-Tellez, B., Garcia-Rivero, Y., Alcantara, JMA., Acosta, FM., Amaro-Gahete, FJ., Llamas-Elvira, JM., Ruiz, JR. Brown adipose tissue and skeletal muscle ^{18}F-FDG activity after a personalized cold exposure is not associated with cold-induced thermogenesis and nutrient oxidation rates in young healthy adults. ***Front Physiol***, in press.

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13. Ruiz JR, Martinez-Tellez B, **Sanchez-Delgado G**, Osuna-Prieto FJ, Rensen PCN, Boon MR. Role of human brown fat in cardiovascular disease: Strategies to turn up the heat. ***Prog Cardiovasc Dis***. 2018
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29. Ruiz JR, **Sanchez-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: 590–1.
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32. Suárez Cadenas E, Cárdenas D, **Sanchez-Delgado G**, Perales JC. The Hidden Cost of Coaching: Intentional Training of Shot Adequacy Discrimination in Basketball Hampers Utilization of Informative Incidental Cues. ***Percept Mot Skills*** 2015; 120: 139–158.
33. 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; 49: 972–3.
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35. Cárdenas D, Ortega E, Llorca J, Courel J, **Sanchez-Delgado G**, Piñar MI. Motor characteristics of fast break in high level basketball. ***Kinesiology*** 2015; 47: 208–214.
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fitness in preschool children: adaptation of the 20 metres shuttle run test]. ***Nutr Hosp*** 2014; 30: 1333–43.
37. Llorca-Miralles J, **Sanchez-Delgado G**, Piñar MI, Cárdenas D, Perales JC. Basketball training influences shot selection assessment: a multi-attribute decision-making approach. ***Rev Psicol Deport*** 2013; 22: 223–226.
38. Perales JC, Cardenas D, Pinar MI, **Sanchez G**, Courel J. Differential effect of incidental and intentional instruction in learning about decision-making conditions when shooting in basketball. ***Rev Psicol Deport*** 2011; 20: 729-745.

Invited conferences

1. **Sanchez-Delgado G**. La evaluación del jugador y del juego como punto de partida para el diseño de programas de entrenamiento condicional en Pádel: una propuesta aplicada. I Congreso Nacional de Investigación en Pádel. Granada, 5 de March 2015.
2. **Sanchez-Delgado G.** La evaluación del jugador y del juego como punto de partida para el diseño de programas de entrenamiento condicional en Pádel: una propuesta aplicada. I Congreso Nacional de Investigación en Pádel. Granada, 5 de March 2015.
3. **Sanchez-Delgado G.** Martinez-Tellez B. Taller: Entrenamiento de Fuerza en función de la pérdida de potencia. I Congreso Nacional de Investigación en Pádel. Granada, 5 de March 2015.
4. **Sanchez-Delgado G**, et al. Role of human brown fat in cardiovascular disease: Strategies to turn up the heat. XXXIX Congreso de la Sociedad Española de Ciencias Fisiológicas. Cádiz, España. 18-21 September, 2018.

Accepted congress communications as first author

1. **Sanchez-Delgado G**, Garcia Rivero Y, Rodríguez-Perez L, Martinez-Tellez B, Alcantara JMA, Amaro-Gahete FJ, Merchan ME, Martinez-Avila WD, Muñoz-Hernandez V, Arias-Tellez MJA, Ramirez Navarro A, Ruiz JR. Association of brown adipose tissue, skeletal muscle glucose uptake and supraclavicular skin temperature, with cold-induced thermogenesis and nutrient oxidation rates. 25th European Congress on Obesity. Vienna, Austria. 23-26 May, 2018.
2. **Sanchez-Delgado G**, Alcantara JMA, Garcia Rivero Y, Martinez-Tellez B, Acosta FM, Amaro-Gahete FJ, Ruiz JR. Cold-induced thermogenesis exhibits a diurnal biorhythm in adults. 4th International Recent Advances and Controversies in Measurement of Energy Metabolism (RACMEM) Conference. Fribourg, Switzerland. 20-22 October, 2017.
3. **Sanchez-Delgado G**, Martinez-Tellez B, Garcia-Rivero Y, Alcantara JMA, Xu H, Ortiz-Alvarez L, Acosta FM, Merchan E, Amaro-Gahete FJ, Olza J, Ruiz JR. (2017). Brown adipose tissue is not associated with bone mineral density in young healthy adults. EMBO (European Molecular Biology Organization) workshop: Brown adipose tissue. Sitges, Spain. 24-27 May 2017.
4. **Sanchez-Delgado G**, Martinez-Tellez B, Garcia-Rivero Y, Muñoz-Hernandez V, Martinez-Avila WD, Ruiz JR. (2017). El tejido adiposo pardo no se asocia con la densidad mineral ósea en adultos jóvenes sanos. II Jornadas de Investigadores en Formación: Fomentando la interdisciplinariedad. Universidad de Granada. Granada, Spain. 17-19 May 2017.
5. **Sanchez-Delgado G**, Martinez-Tellez B, Alcántara JMA, Muñoz-Hernandez V, Ramirez-Navarro A, Sanchez-Sanchez R, Olza J, Aguilera CM, Gil A, Ruiz JR. (2016) Associations of shivering threshold with cardiorespiratory fitness, physical activity and body composition in young adults: preliminary results of the ACTIBATE Study. European Obesity Summit 2016. Gothenburg, Sweden.

6. **Sanchez-Delgado G**, Alcántara JMA, Martinez-Tellez B, Ruiz JR. (2016). Efecto de un programa de ejercicio combinado de 14 semanas de duración sobre la composición corporal en adultos jóvenes: Resultados preliminares del estudio ACTIBATE. I Jornadas de Investigadores en Formación: Fomentando la interdisciplinariedad. Universidad de Granada. Granada, Spain. 18-20 May 2016.
7. **Sanchez-Delgado G**, Cadenas-Sánchez C, Mora-González J, Martínez-Téllez B, Chillón P, Löf M, Ortega FB, Ruiz JR. "Assessment of handgrip strength in preschool children aged 3 to 5 years". Symposium EXERNET: Red española de investigación en ejercicio físico y salud. Granada, Spain. 7-8 2014 November.
8. **Sanchez-Delgado G**, López-Viseas J, Cárdenas D, Perales JC. Prior mood state modulates the psychological effects of exercise: I. Acute affective effects. VII Congreso Internacional de la Asociación Española de Ciencias del Deporte. Granada, Spain. 15-17 November 2012.
9. **Sanchez-Delgado G**, López-Viseas J, Cárdenas D, Perales JC. Prior mood state modulates the psychological effects of exercise: II. Perceived exertion. VII Congreso Internacional de la Asociación Española de Ciencias del Deporte. Granada, Spain. 15-17 November 2012.
10. **Sanchez-Delgado G**, Courel, J, Estevez-López F, Ortega E, Piñar MI, Cárdenas D. Descriptive study of fastbreak motor variables at top level basketball. Comparative analysis between winners and losers. VII Congreso Internacional de la Asociación Española de Ciencias del Deporte. Granada, Spain. 15-17 November 2012.
11. **Sanchez-Delgado G.** Conde-González J, Perales JC, Pinar MI, Cárdenas D, De Teresa-Galván C, Ruiz JR. Acute cognitive workload hampers subjective recovery, but does not accelerate exhaustion in a maximal effort test on a treadmill. European Congress of Sport Science. Bruges, Belgium. 4-7 July 2012.
12. **Sanchez-Delgado G.** Conde-González J, Cárdenas D, Perales JC, Piñar López MI, De Teresa-Gálvan C, Ruiz JR. (2012) La interacción de la carga de trabajo física y mental en la percepción del esfuerzo durante y después de un ejercicio físico hasta el agotamiento en tapiz rodante. XIII Congreso Nacional y I Foro Mediterraneo de Psicología de la Actividad Física y el Deporte. Murcia, Spain. 21-24 March 2012.

Other merits

2010	University of Granada outstanding pupil award (1 of 160 given) in 2009/2010 academic course.
2013	Certified personal trainer. National Strength and Conditionining Association.
2015-	Lecturer in the degree of Sports Sciences. University of Granada.
2014-	Lecturer in the master's degree in personal training. University of Granada.
2016-	Lecturer of the National Strength and Conditionining Association (NSCA-Spain).
2012-	Co-author of 61 congress communications (including national and international conferences).

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A ojos de este doctorando, obtener el título de Doctor al que con esta Tesis se aspira, no supone sino uno de los primeros pasos de una vocacional carrera científica. A mi juicio, ejercer como investigador científico debe ser una de las más nobles ocupaciones humanas, contribuyendo notablemente a llenar de significado la experiencia vital. Vivir guiado por la curiosidad y hacer del aprendizaje y el descubrimiento el centro sobre el que todo gira, es, en mi corta experiencia, una de las formas más efectivas de conciliarnos con nuestra naturaleza humana. Por ello, el hito que representa la obtención del título de Doctor y el estar en disposición de iniciar una carrera científica es para mí un motivo de enorme alegría. Soy muy consciente de que no podría disfrutar hoy de este enorme privilegio sin haber vivido en unas circunstancias tan extraordinariamente favorables, sin haber recibido tanta ayuda y apoyo, y sin haber estado rodeado, desde hace mucho tiempo, de gente extraordinaria. Es por lo tanto un hecho de profunda justicia que las últimas páginas de este documento se llenen con el sincero reconocimiento a aquellas personas a las que en mayor o menor medida debo el privilegio de presentar esta Tesis Doctoral y que son directamente responsables del enorme aprendizaje que he adquirido en el proceso de su elaboración.

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In 2009, brown adipose tissue (BAT) was discovered to be present and active in human adults. Since then, BAT has been regarded as a promising target for therapies against obesity and related diseases. However, whether BAT is able to significantly influence human energy balance and metabolism regulation is still unclear. Moreover, there is biological base to hypothesize that BAT activation and/or recruitment takes place in response to physical exercise, possibly being one of the still unknown mechanisms by which exercise exert important beneficial effects on human health.

The main aims of this Doctoral Thesis are to study the role of human BAT in energy balance regulation, the relationship between BAT and body composition, the role of BAT in the development of the metabolic syndro - me in overweight or obese patients, and the effect of exercise on BAT volume and activity.