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# Energy Expenditure and Macronutrient Oxidation in Response to an Individualized Nonshivering Cooling Protocol

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**Objective:** This study aimed to describe the energy expenditure (EE) and macronutrient oxidation response to an individualized nonshivering cold exposure in young healthy adults.

**Methods:** Two different groups of 44 (study 1: 22.1 [SD 2.1] years old, 25.6 [SD 5.2] kg/m$^2$, 34% men) and 13 young healthy adults (study 2: 25.6 [SD 3.0] years old, 23.6 [SD 2.4] kg/m$^2$, 54% men) participated in this study. Resting metabolic rate (RMR) and macronutrient oxidation rates were measured by indirect calorimetry under fasting conditions in a warm environment (for 30 minutes) and in mild cold conditions (for 65 minutes, with the individual wearing a water-perfused cooling vest set at an individualized temperature adjusted to the individual's shivering threshold).

**Results:** In study 1, EE increased in the initial stage of cold exposure and remained stable for the whole cold exposure ($P<0.001$). Mean cold-induced thermogenesis (9.56 ± 7.9 kcal/h) was 13.9% ± 11.6% of the RMR (range: −14.8% to 39.9% of the RMR). Carbohydrate oxidation decreased during the first 30 minutes of the cold exposure and later recovered up to the baseline values ($P<0.01$) in parallel to opposite changes in fat oxidation ($P<0.01$). Results were replicated in study 2.

**Conclusions:** A 1-hour mild cold exposure individually adjusted to elicit maximum nonshivering thermogenesis induces a very modest increase in EE and a shift of macronutrient oxidation that may underlie a shift in thermogenic tissue activity.

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## Study Importance

### What is already known?

- A decade ago, brown adipose tissue was reported to be present and metabolically active in adult humans. Since then, nonshivering mild cold exposure has been considered a potential therapeutic tool in obesity management.
- Chronic exposure to nonshivering cold stimulus appears to improve energy metabolism and metabolic health.

### What does this study add?

- A mild cold exposure at a temperature adjusted to elicit maximum nonshivering thermogenesis induces a modest increase in energy expenditure, which is maintained constantly over an hour.
- A metabolic shift was observed by which fat oxidation (FATox) increased in parallel to a decrease in carbohydrate oxidation (CHOox) at the beginning of cold exposure. Later, an inverse tendency appeared, by which CHOox increased and FATox slightly decreased.

### How might these results change the direction of research?

- This study suggests that mild cold exposure is not a feasible tool to induce negative energy balance in humans. However, pharmacological or nutraceutical continuous induction of maximum nonshivering thermogenesis might be a feasible target for weight loss.

## Introduction

Because brown adipose tissue (BAT) was shown to be present and metabolically active in adult humans in 2009 (1-4), cold-induced thermogenesis (CIT) has received considerable attention as a possible target for stimulating BAT and energy expenditure (EE), helping weight control and counteracting obesity (5). Indeed, several independent groups have reported that chronic exposure to nonshivering cold stimulus seems to enhance energy metabolism and metabolic health in healthy individuals (6) and in people with obesity (7) or type 2 diabetes mellitus (8).

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It is noteworthy that it remains unclear whether human BAT-related health benefits are explained by increases in EE or by other mechanisms beyond energy balance (9,10). Moreover, there is an open debate regarding the relative contribution of BAT to CIT, in comparison with other thermogenic tissues such as skeletal muscle or white adipose tissue (11).

CIT is produced by both nonshivering and shivering mechanisms (12). Shivering thermogenesis is the EE necessary to cover involuntary skeletal muscle contractions in response to cold, whereas nonshivering thermogenesis refers to the energy-consuming processes not dependent on muscle contraction, such as uncoupling respiration in brown adipocytes' mitochondria (13). Although some have reported that shivering thermogenesis can increase EE up to fivefold above resting metabolic rate (RMR), its uncomfortable nature, together with the loss of motor coordination, makes it a poorly tolerated stimulus and, therefore, an implausible option to be used in clinical settings (13). Moreover, nonshivering thermogenesis produces moderate increases of EE, and estimations have reported a range between 0% and 30% of the RMR in young healthy adults (13). Moreover, it has been argued that nonshivering thermogenesis may be comfortable enough to be considered a possible tool in the prevention and treatment of obesity (13). Of note, shivering and nonshivering thermogenesis does not appear to occur in sequential phases but more as parallel phenomena (i.e., even with mild cold stimulation, shivering thermogenesis seems to contribute to CIT) (14), and the relative contribution to CIT can vary within individuals (15).

There is a large interindividual variation in nonshivering thermogenesis and cold tolerance. Therefore, to study the responses to mild cold exposure, there is a need to individualize the cold stimulus to the individual's cold tolerance (16,17). Van der Lans et al. (16) proposed to first assess a shivering threshold (i.e., the 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 (18) or cold perception adjustment (19), the lowest tolerable temperature above the shivering threshold has been broadly accepted and used as a valid approach to induce BAT activation and CIT (17,20-23). Some studies have, however, shown that skeletal muscle thermogenesis takes place with very mild cold stimuli before shivering can be observed (14). As a result, the shivering threshold approach may not fully exclude skeletal muscle shivering thermogenesis.

Research has traditionally spent more attention on the study of shivering thermogenesis (24), and important gaps remain regarding the human physiological responses to mild cold exposure in terms of EE and metabolic fuel selection (12). We hypothesized that a mild cold exposure designed to elicit nonshivering thermogenesis would exert slight, but clinically meaningful if maintained over time, modifications of EE and metabolic fuel selection. Thus, the present study aimed to describe the EE and macronutrient oxidation response to an individualized nonshivering cold exposure in young healthy adults.

# Methods

## Participants

Two different cohorts took part in this study (Table 1). For the first cohort (hereafter called study 1), 63 participants (45 female) were included in the study, all of them being part of the ACTIBATE study (25). However, only 44 out of 63 were included in the statistical analyses (Figure 1). Study 1 was conducted in October and November of 2016. For study 2, 13 participants were recruited and evaluated between December 2017 and January 2018. Inclusion criteria in both studies were as follows: <35 years old, reports being healthy, does not smoke or take any medication, has had a stable body weight (<3-kg change) during the past 3 months, and is not regularly exposed to cold. The study and written informed consent considered the last revision of the Declaration of Helsinki and were approved by the human research ethics committee of the University of Granada (No. 924) and of the Servicio Andaluz de Salud (Centro de Granada, *Comité de Ética de la Investigación*-Granada).

We used a whole-body dual-energy x-ray absorptiometry scan (Discovery Wi, Hologic, Inc.) to assess body composition, whereas a Seca scale and stadiometer (model 799, Electronic Column Scale) were used to measure weight and height, respectively.

## Conditions prior to the study days

Participants reported to the research center on two separate occasions. During the first visit, shivering threshold was assessed, whereas during the second visit, CIT was measured. They were asked to sleep as usual, to avoid both moderate (24 hours) and vigorous (48 hours) physical activity prior to the testing days, and to commute by motorized vehicle.

**TABLE 1** Participant characteristics

| | Study 1, CIT analyses ($n=44$) | Study 1, NUTox analyses ($n=18$) | Confirmatory study (study 2, $n=13$) |
|---|---|---|---|
| Sex, *n* women (%) | 29 (65.9) | 13 (72.2) | 6 (46.2) |
| Age, y | 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/d | 1,565 (278) | 1,554 (227) | 1,484 (286) |
| RER | 0.862 (0.054) | 0.842 (0.048) | 0.833 (0.036) |
| Cooling-vest water temperature, °C | 8.4 (3.5) | 9.2 (3.8) | 12.1 (3.1) |

Data are mean (SD), except for sex.
CIT, cold-induced thermogenesis; NUTox, macronutrient oxidation rates; RER, resting respiratory exchange ratio; RMR, resting metabolic rate.

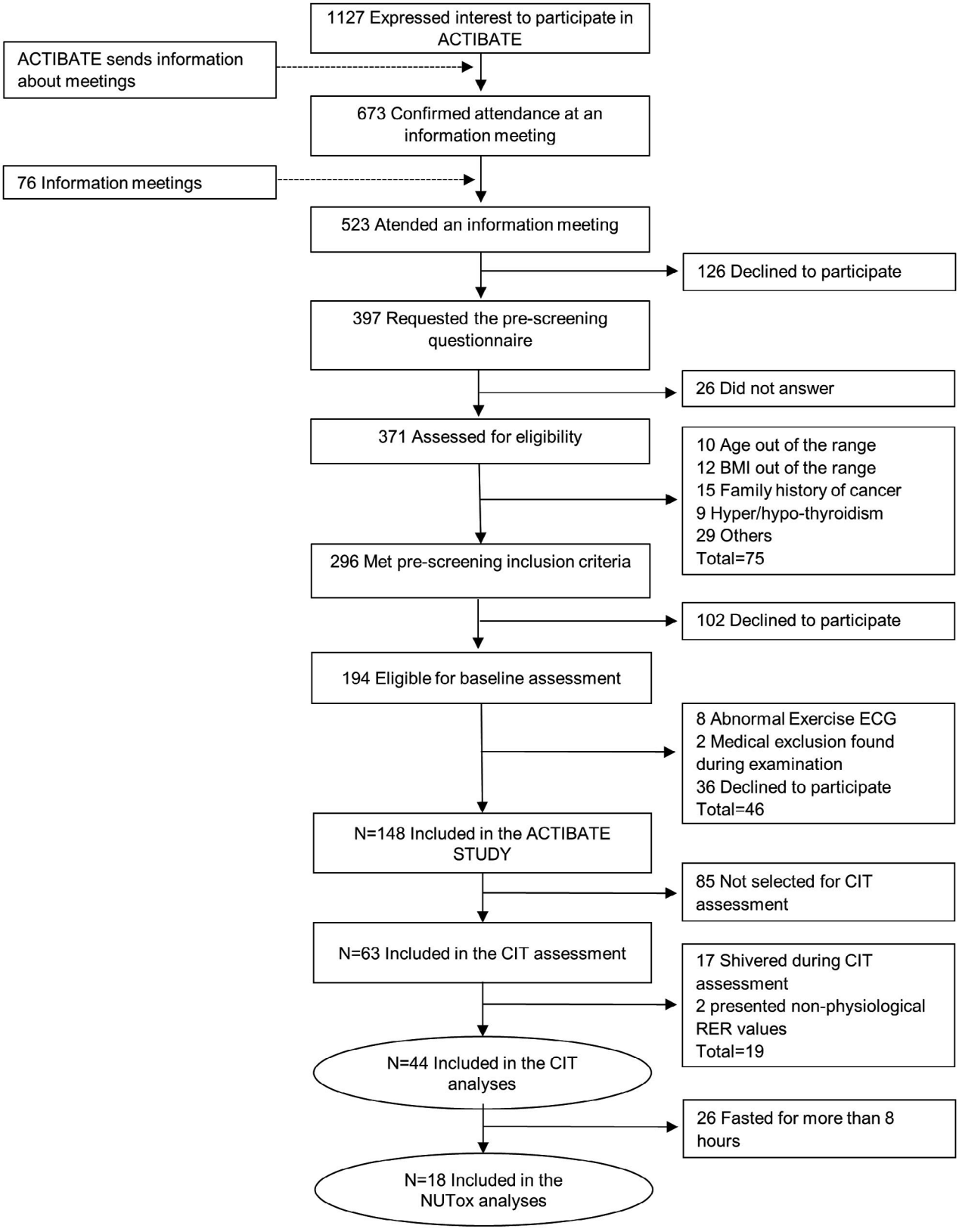


**Figure 1** Participant flowchart. ACTIBATE, Activating Brown Adipose Tissue Through Exercise study; CIT, cold-induced thermogenesis; ECG, electrocardiogram; NUTox, nutrient oxidation; RER, respiratory exchange ratio.

In study 1, participants fasted for at least 6 hours (9 ± 3.7 hours). Only those participants strictly following the fasting time indications (i.e., between 6 and 8 hours; $n = 18$) were included in the macronutrient oxidation rate analyses (26,27) (Table 1). In study 2, participants were instructed to consume a standardized meal (35% of estimated total EE; boiled rice, tomato sauce, and omelet) 10 hours before the CIT assessment. Moreover, they were instructed to collect all urine from the time of the standardized meal to the CIT assessment initiation (i.e., 10-hour urine production). Another urine sample was collected immediately after the CIT assessment.

## Shivering threshold test (day 1)

The shivering threshold assessment methodology has been extensively described elsewhere (23,28). In brief, after voiding, the participants dressed with standardized clothes (clothing insulation value: 0.20). They then entered a warm room (22.1 ± 1.6 °C) where they remained seated for 30 minutes before entering a cold room (19.8 ± 0.5 °C) where they dressed with a temperature-controlled water-perfused cooling vest (Polar Products Inc.). The vest circulating water temperature was initially set at 16.6 °C and was decreased every 10 minutes until 3.8 °C was reached or until shivering occurred. Shivering was determined visually and was self-reported. The vest circulating water temperature when shivering started was considered as the shivering threshold.

## CIT and cold-induced macronutrient oxidation rates (day 2)

Participants returned to the laboratory (the same time of day as before) 5 to 7 days (study 1) or 2 days (study 2) after the shivering threshold test. After voiding, they dressed in the same standardized clothes and entered a warm (23.2 ± 0.7 °C) room. Before EE was assessed, all participants reclined in a bed for 20 minutes. Later, their RMR was assessed over 30 minutes, following current methodological guidelines (29). Immediately after, the participants walked into the cold room (19.7 ± 0.4 °C) and dressed in the cooling vest, which was set at 4 °C above the participant's shivering threshold. They lay in a reclined bed while the CIT assessment was carried out for two 30-minute periods, separated by a 5-minute pause. For assessing RMR and CIT, ventilatory gas exchange was collected using a neoprene face mask hooked up to a CCM Express or Ultima CardiO2 metabolic cart (MCG Diagnostics) (30,31). Flow, at the beginning of every test day, and gas analyzers, before every 30-minute assessment, were calibrated following the manufacturer's instructions.

In study 1, despite the careful assessment of the shivering threshold, 17 (16 women) out of 63 participants shivered during the CIT assessment. Those individuals were excluded from further statistical analysis. Moreover, two males were also excluded from the analyses for presenting respiratory exchange ratio values higher than 1.1 or lower than 0.7 at any measured time point (29) (Figure 1).

## CIT and macronutrient oxidation rate estimation

Oxygen consumption ($VO_2$) and carbon dioxide production ($VCO_2$) were downloaded from the metabolic cart software (BreezeSuite, version 8.1.0.54 SP7; MCG Diagnostics) every minute. For all 30-minute recording periods, we discarded the first 5-minute record. We then selected the most stable 5-minute period among the remaining records (25 minutes) to be considered as the RMR, as previously described (30,32). For calculating CIT, data were averaged every 5 minutes. In addition, we divided the CIT record into four parts and selected the most stable 5-minute period within each part. Finally, we calculated the area under the curve (trapezoidal rule) using the four selected 5-minute periods and the RMR, expressing it as a percentage of the RMR. We have previously published that this method for data analyses resulted in more plausible estimations when compared with other methods for data analysis (33). The Weir abbreviated equation was used to estimate EE (Equation 1) (34), whereas Frayn equations (35) (not taking into account any urinary nitrogen in study 1) were used for estimating carbohydrate oxidation (CHOox) (Equation 2) and fat oxidation (FATox) (Equation 3).

$$\mathrm{EE\,(kcal/min)} = 3.941 \times \mathrm{VO_2\,(L/min)} + 1.106 \times \mathrm{VCO_2\,(L/min)} - 2.17 \times N\,\mathrm{(g/min)} \quad (1)$$

$$\mathrm{CHOox\,(g/min)} = -3.21 \times \mathrm{VO_2\,(L/min)} + 4.55 \times \mathrm{VCO_2\,(L/min)} - 2.87 \times N\,\mathrm{(g/min)} \quad (2)$$

$$\mathrm{FATox\,(g/min)} = 1.67 \times \mathrm{VO_2\,(L/min)} - 1.67 \times \mathrm{VCO_2\,(L/min)} - 1.92 \times N\,\mathrm{(g/min)} \quad (3)$$

In study 2, we measured total urine volume and urea concentration in both urine samples. Thereafter, we estimated nitrogen urine levels from the urea concentration (Spinreact, catalog No. 283-17) following the equation $N$ (grams per liter) = 0.0065 × urea (mg/dL) + 1.2598, which was obtained from a linear regression including measured urea and nitrogen concentrations (Kjeldahl method (36)) from an independent cohort of young healthy adults ($n = 19$, 16 women; 21.87 ± 2.05 years old; 24.87 ± 3.71 kg/m$^2$). Protein oxidation (grams per minute) was calculated as $N$ (grams per minute) × 6.25 (37).

## Statistical analysis

Repeated-measures ANOVA with Bonferroni post hoc corrections was used to test differences in EE, CHOox, and FATox across time. Paired $t$ tests were used to compare protein oxidation before and during the cold stimulus.

The analyses were conducted using SPSS Statistics version 21.0 (IBM Corp). Figures were created using GraphPad Prism (version 8.3.1). The level of significance was set at <0.05.

# Results

Mild cold exposure significantly increased EE ($P < 0.001$; Figure 2A) from the beginning of cold exposure and EE remained stable from that moment until the end of the mild cold exposure. The mean overall CIT estimation was 13.9% ± 11.6% of the RMR (range: −14.8% to 39.9% of the RMR; Figure 2B). When translated to cumulative EE (i.e., the total amount of energy above the RMR expended during the whole 65-minute cold exposure), it resulted in 9.56 ± 7.9 kcal.

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 ). The peak FATox was observed at 30 minutes of cold exposure ($P < 0.001$; Figure 2D). FATox progressively increased until minute 30, and later on, there was a decrease. All results were similar in men and women and in individuals with BMI < 25 kg/m$^2$ and individuals with BMI > 25 kg/m$^2$ (data not shown). The mean overall cold-induced CHOox was −7.3% ± 22.5% of basal CHOox (range: −36.4% to 34.31% of basal CHOox) and mean overall cold-induced FATox was 53.6% ± 90.9% of basal FATox (range: −33.9% to 382.1% of basal FATox).

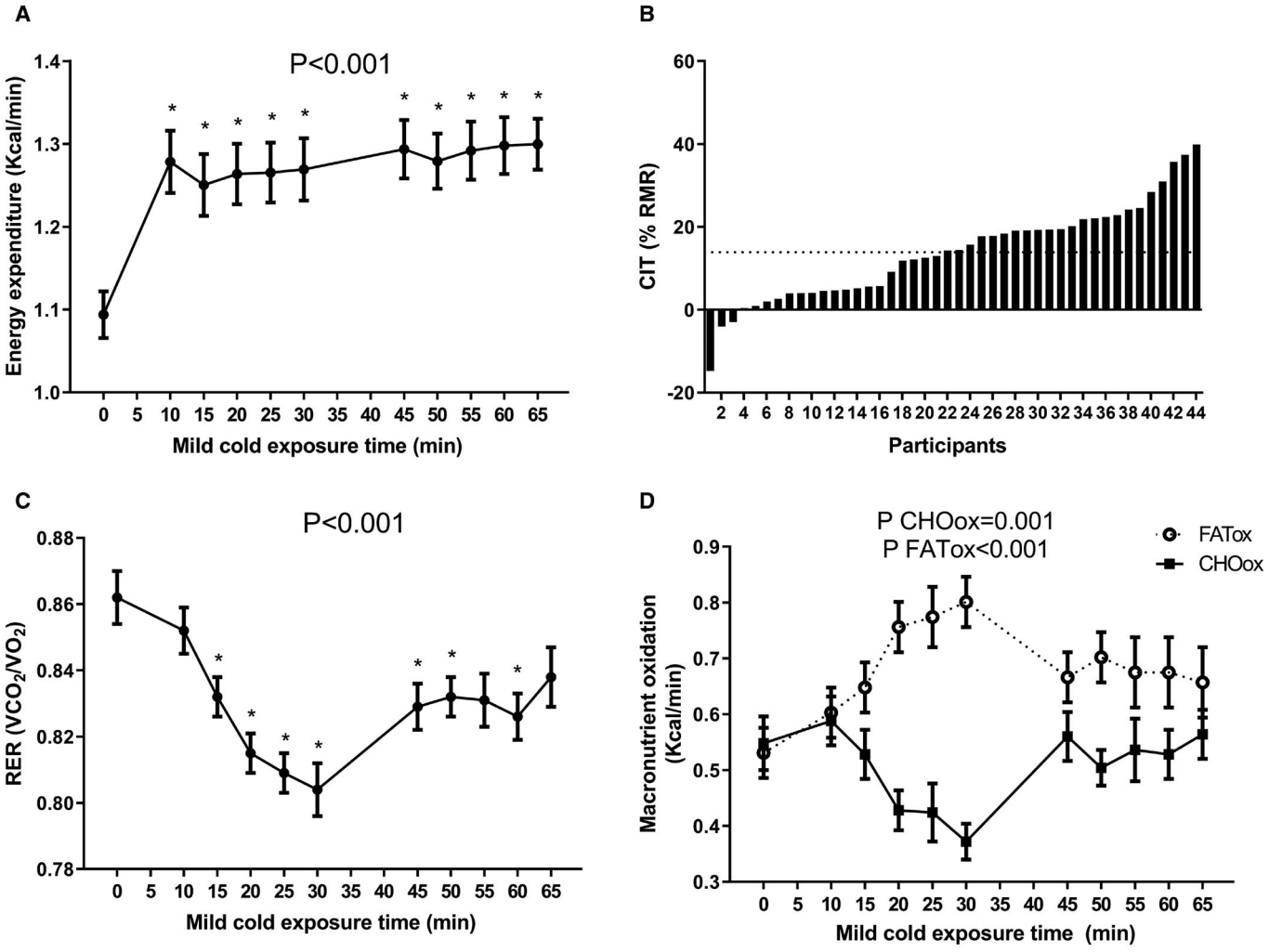


**Figure 2** Cold-induced thermogenesis (CIT) and macronutrient oxidation rates (study 1). (**A**, **C**-**D**) Minute 0 represents resting metabolic rate (RMR). Values are the mean and SEM. *P* values were calculated using repeated-measure ANOVA. Asterisks (*) represent values significantly different from the RMR. (**B**) Histogram representing overall CIT (each column representing one individual's CIT). Dashed line represents mean CIT. CHOox, carbohydrate oxidation; FATox, fat oxidation; RER, respiratory exchange ratio; $VCO_2$, carbon dioxide production $VO_2$, oxygen consumption. Panel A adapted with permission from Sanchez-Delgado et al. (33).

Figure 3 shows EE and macronutrient oxidation rates during a mild cold exposure in study 2. Cold exposure increased EE, as in study 1 ($P>0.001$; Figure 3A). The effect of cold exposure on both CHOox and FATox (Figure 3D) was also similar to that in study 1. Finally, Figure 4 shows the estimated protein oxidation before (10 hours) and during the mild cold exposure in study 2. Mild cold exposure significantly increased protein oxidation ($0.91\pm0.38$ g/min during the cold exposure vs. $0.54\pm0.29$ g/min for the 10 hours preceding cold exposure; $P=0.005$).

## Discussion

We show that a 1-hour individualized mild cold exposure produced a moderate increase in EE ($9.56\pm7.9$ kcal/h) 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 to the baseline value at 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 reinforces the findings. Our results show a modest EE increase during mild cold exposure, which is consistent with findings of previous studies (12). Our data also show a previously unreported shift in the nutrient oxidation rates for sustaining CIT during the first phase of mild cold exposure (first 30 minutes). This finding might have important implications for the understanding of physiological processes underlying human nonshivering thermogenesis.

### Mild cold exposure induces moderate increases in EE

In study 1, some individuals showed negative values of CIT (i.e., lower EE in cold than at RMR), whereas others experienced a 39% increase over the RMR, which is consistent with the huge interindividual variability (38,39) that has been reported in previous studies. Whether

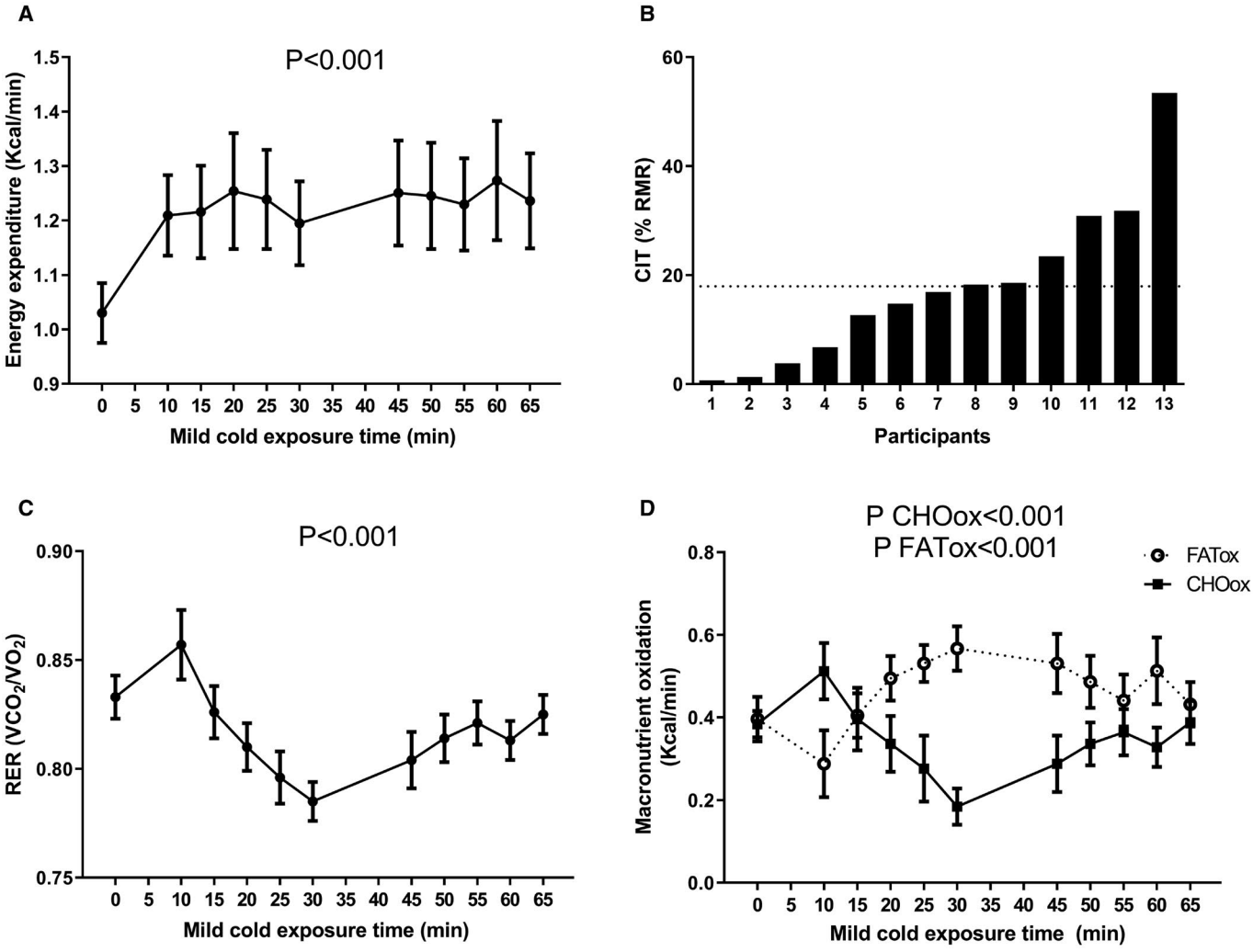


**Figure 3** Cold-induced thermogenesis (CIT) and macronutrient oxidation rates (study 2). (**A**, **C**-**D**) Minute 0 represents resting metabolic rate. Values are the mean and SEM. *P* values were calculated using repeated-measure ANOVA. (**B**) Histogram representing overall CIT (each column representing one individual's CIT). Dashed line represents mean CIT. CHOox, carbohydrate oxidation; FATox, fat oxidation; RER, respiratory exchange ratio; $VCO_2$, carbon dioxide production $VO_2$, oxygen consumption.

nonshivering cold exposure induces a stable EE change from the beginning of the exposure is unclear (40). We observed that when applying an individualized cooling protocol at a temperature near the shivering threshold, maximum nonshivering thermogenesis appeared to be elicited from the beginning of the cold exposure. This constant thermogenic rate is consistent with findings from other studies analyzing the EE elicited by shivering (41,42). Moreover, it is not clear whether the magnitude of nonshivering thermogenesis is able to induce a significant negative energy balance (14,43). We observed that even when applying a protocol designed to elicit maximum nonshivering thermogenesis, all participants presented an EE below 1.4 metabolic equivalents (METs; i.e., 40% of RMR increase). Taking into account that even a very-low-intensity exercise, such as walking, can elicit 2 to 3 METs, together with the fact that cold exposure may induce a hyperphagic response (44), it seems that cold exposure at temperatures eliciting low shivering and maximum nonshivering thermogenesis is not an efficient stimuli to induce negative energy balance. It should also be noted that this mild cold stimulus produced a considerable burden and discomfort for participants, despite only 9.56 ± 7.9 kcal being burned (above the RMR) after an hour in this uncomfortable situation. Nonetheless, if maximum nonshivering thermogenesis were induced by pharmacological or nutraceutical agents (45,46), instead of cold exposure, and were continuously elicited over 24 hours, our data would translate into 212 ± 175 kcal/d. Importantly, recent studies using radiotracers for the quantification of BAT EE have estimated that, if maximally and continually activated, human BAT could account for ≈15 kcal/d (20,47,48). This is less than 10% of the 24-hour CIT estimation made in our study, which suggests that tissues other than BAT (e.g., skeletal muscle) may represent better targets for stimulating nonshivering thermogenesis in humans (5). Nonetheless, alternative mechanisms beyond energy balance (9,10) have been suggested to mediate the health-promoting effects of BAT and chronic cold exposure (6-8). Therefore, mild cold exposure and/or pharmacological BAT activation or recruitment might not

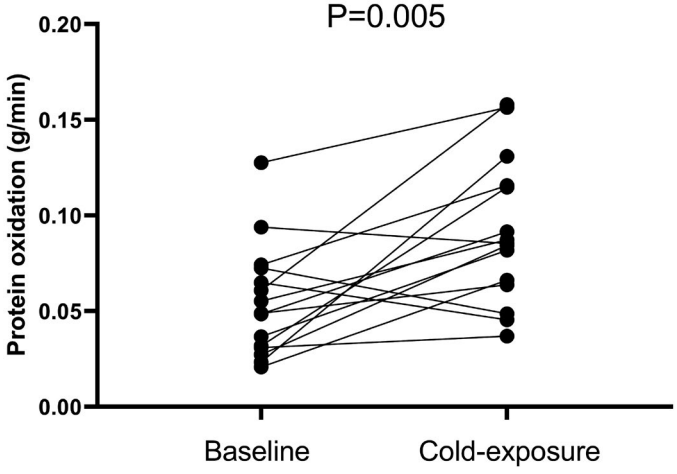


**Figure 4** Protein oxidation before (10 hours) and during mild cold exposure in study 2. *P* values are from paired *t* tests.

be discarded as possible therapeutic targets including insulin-sensitizing effects.

### Mild cold exposure induces shift in 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) (12,41,42); 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 (12). Consequently, fatty acids are the most sustainable fuel for thermogenic purposes. FATox is the predominant substrate for both nonshivering and shivering thermogenesis (12). Therefore, it is biologically plausible that a shift to FATox is produced when thermogenic needs are not maximized, such as at the beginning of a cold exposure.

BAT is not the only contributor responsible for nonshivering thermogenesis in humans. Skeletal muscle possibly contributes to nonshivering thermogenesis to an even larger extent (14,20). Although both BAT and skeletal muscle preferentially use fatty acids as fuel in nonshivering situations, BAT is probably more FATox-preferential, as more than 90% of its energy consumption relies on FATox (49,50), whereas skeletal muscle presents a more balanced nutrient uptake (12). Therefore, it is plausible to speculate that BAT is the main contributor to CIT at initial stages of mild cold exposure, whereas the muscle contribution to CIT would increase progressively, therefore balancing the contribution of CHOox and FATox. This hypothesis is supported by recent studies showing that BAT is rapidly activated upon cold exposure and seems to stabilize after 35 minutes of cold exposure (51-53). Moreover, we previously reported a high prevalence of BAT in study 1 (88% BAT-positive, BAT volume = 94.4 ± 59.6 mL), as measured by static $^{18}$F-flurodeoxyglucose positron emission tomography/computed tomography scanning after 2 hours of personalized cold exposure (23,54); therefore, BAT thermogenesis was likely induced during the mild cold exposure. New studies examining the contribution of both tissues, BAT and skeletal muscle, from the very beginning of the cold exposure are needed to confirm such hypothesis.

Protein oxidation was assessed only in study 2, in which we observed a significant increase in response to mild cold exposure. A recent study in both mice and humans showed that upon cold exposure, BAT significantly increased the uptake and oxidation of branched-chain amino acids (BCAAs) (55). Importantly, blunting BCAA oxidation in BAT significantly affected the BAT thermogenic capacity in mice (55). Therefore, the increase of protein oxidation observed in our study might be explained by the increased uptake and oxidation of BCAAs in BAT. Such a statement is quite speculative, as we did not specifically measure BCAA oxidation in study 2. Further studies are needed to test whether there is an association between BAT activity and cold-induced protein oxidation in humans.

In agreement with findings from previous studies (12), we observed a considerably high interindividual variability in cold-induced macronutrient oxidation rates. In human studies, different patterns of shivering (i.e., muscle recruitment) explain most of the interindividual variability in cold-induced macronutrient oxidation rates (12,41,42). Because the protocol we applied is considered to result in a low-shivering contribution to CIT, different patterns of shivering may not explain such a large interindividual variability. Alternatively, interindividual differences in tissues' (BAT, skeletal muscle, and white adipose tissue) relative contribution to CIT (i.e., proportion of CIT being produced by each tissue) might partially explain such a high interindividual difference (5).

### Limitations

In study 1, CHOox and FATox rates were calculated without considering protein oxidation, as we did not measure urinary nitrogen excretion. In addition, in study 1, we did not strictly control the fasting period or previous meal content. However, we performed a confirmatory study (i.e., study 2) analyzing protein oxidation and strictly controlling the fasting time and previous meal content, and similar results were found. Second, visually detected or self-reported shivering is likely to occur after electrical muscle activity (40). Therefore, the absence of electromyographic recording might have resulted in an underestimation of the shivering threshold temperature. Moreover, despite excluding participants presenting detectable shivering during the CIT assessment, we cannot dismiss the possible presence of shivering thermogenesis that was not visually detectable or self-noted (14). Nonetheless, it is probable that nonshivering thermogenesis was the predominant form of CIT in the included participants. In addition, it should be noted that the study sample was entirely composed of young (18-32 years) healthy individuals, and the results thus cannot be generalized to older or unhealthy individuals. Finally, it should be noted that the reliability of the indirect calorimeters used in this study was not optimal (31). This probably explains the presence of nonphysiological values obtained in 2 out of our 63 participants enrolled in study 1.

## Conclusion

A mild cold exposure at a temperature adjusted to elicit maximum nonshivering thermogenesis induces a very modest increase in EE (<40% of RMR; ≈1.4 METs), which is maintained constantly over an hour. The cumulative cold-induced EE over an hour of mild cold exposure (9.6 ± 7.9 kcal/h) is probably insufficient to induce negative energy balance in humans. However, we cannot exclude the possibility that sustained induction of maximum nonshivering CIT (212 ± 175 kcal/d), which might be achieved by pharmacological or nutraceutical agents,

could be a feasible strategy for weight loss. Interestingly, we found a metabolic shift in 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 the main role in CIT during the first stages of cold exposure could be different from those playing the main role in CIT after 30 minutes of cold exposure.O

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**Author contributions:** GSD, JMAA, FMA, BMT, and JRR designed the research; GSD, JMAA, FMA, BMT, FJAG, and EMR conducted the research; GSD and JMAA analyzed the data; GSD, ML, ER, and JRR wrote the manuscript; GSD and JRR had primary responsibility for the final content; and GSD, JMAA, FMA, BMT, FJAG, EMR, ML, IL, ER, and JRR discussed the results and approved the final version of the manuscript.

**Clinical trial registration:** ClinicalTrials.gov identifier NCT02365129.

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