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

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ABSTRACT

Background & Aims: Since the discovery of active brown adipose tissue in human adults, non-shivering cold-induced thermogenesis (CIT) has been regarded as a promising tool to combat obesity. However, there is a lack of consensus regarding the method of choice to analyze indirect calorimetry data from a CIT study. We analyzed the impact of methods for data selection and methods for data analysis on measures of cold-induced energy expenditure and nutrient oxidation rates.

Methods: Forty-four young healthy adults Caucasians (22.1±2.1 years old, 25.6±5.2 kg/m^2, 29 women) participated in the study. RMR, CIT, and cold-induced nutrient oxidation rates were estimated by indirect calorimetry under fasting conditions during one hour of cold exposure combining air conditioning (19.5-20ºC) and a water perfused cooling vest set at a temperature of 4ºC above the individual shivering threshold. We applied three methods for data selection: (i) time intervals every 5 minutes (*5min-TI*), (ii) the most stable 5-minute period of every forth part of the cold exposure (*5min-SS-4P*), and (iii) the most stable 5-minute period of every half part of the cold exposure (*5min-SS-2P*). Lately we applied two methods for data analysis: (i) area under the curve as a percentage of the baseline RMR (AUC) and; (ii) the difference between EE at the end of the cold exposure and baseline RMR (Last-RMR).

Results: 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. Regarding methods for data selection, *5min-SS-2P* did not allow to observe physiologically relevant phenomena (e.g. metabolic shift in fuel oxidation; P=0.547) due to a lack of resolution. The *5min-TI* and *5min-SS-4P* methods for data selection seemed to be accurate enough to observe physiologically relevant phenomena (all P<0.014), but not comparable for estimating over-all CIT and cold-induced nutrient oxidation rates (P<0.01). Regarding methods for

data analysis, the AUC seemed to be less affected for data artefacts and to be more representative in participants with a non-stable energy expenditure during cold exposure.

Conclusions: The methods for data selection and analysis can have a profound impact on CIT and cold-induced nutrient oxidation rates 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 cold-induced nutrient oxidation rates, while *5min-SS-4P* and AUC should be the method of choice when computing CIT and cold-induced nutrient oxidation rates as a single value.

Keywords: Cold-induced thermogenesis, adaptive thermogenesis, indirect calorimetry, metabolic rate, energy balance, obesity.

63 **ABBREVIATIONS**

64 BAT: Brown adipose tissue.

65 CIT: Cold-induced thermogenesis.

66 RMR: Resting metabolic Rate.

67 SS: Steady state.

68 TI: Time interval.

69 EE: Energy expenditure.

70 AUC: Area under the curve.

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

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

73 DXA: Dual X-ray absorciometry.

74 RER: Respiratory exchange ratio.

75 CHOox: Carbohydrates oxidation.

76 FATox: Fat oxidation.

77 ANOVA: Analyses of variance.

78 5min-TI: mean values of every consecutive 5-minute period.

79 5min-SS-4P: The most stable 5-minute period of every forth part of the cold exposure.

80 5min-SS-2P: The most stable 5-minute period of every half part of the cold exposure.

INTRODUCTION

In simple terms, obesity results from a positive energy balance (i.e. lower energy expenditure than energy intake), and thus, weight loss would be easily achieved inducing a negative energy balance. However, many physiological and behavioral adaptations occur in parallel to caloric restriction, making weight loss unsuccessful in long-term [1]. Currently there are no non-invasive successful strategies to achieve sustainable weight loss, and new strategies have to be explored [1]. During the last decade, brown adipose tissue (BAT) activation has been regarded as a possible solution to the obesity problem [2].

BAT was confirmed to be present and active in adult humans in 2009 [3–6]. Since them, BAT has been considered a promising therapeutic target due to its capacity to oxidize glucose and lipids for heat producing purposes, in a process known as non-shivering thermogenesis. In murine models, BAT thermogenesis can account up to 60% of total energy expenditure [7]. In humans, BAT is much more scarce than in murine [8] and there is an open debate on whether BAT activity can significantly influence human energy expenditure [9,10]. Noteworthy, even assuming the most pessimistic views of BAT potential to contribute to energy expenditure [11–14], non-shivering thermogenesis can be mediated by other tissues, such as withe adipose tissue and skeletal muscle [12,13,15], and seems to be relevant enough to be considered a possible solution to the weight loss maintenance problem [1].

Cold-induced thermogenesis (CIT) can be broadly divided into shivering and non-shivering thermogenesis [16], although both processes can occur concomitantly [12]. CIT and the associated changes in nutrient oxidation rates are commonly measured by indirect calorimetry [17]. Indirect calorimetry data are quite variable minute by minute, and methods for data selection based on steady state (SS) periods are often necessary to

minimize individuals' and instrument's variability [17–22]. Alternatively, selection of predefined (i.e. not considering data variability) time intervals (TI) is commonly made [18]. 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 [23]. Therefore, investigators have used area under the curve calculations (AUC) and/or the difference between EE at the end of cold exposure and baseline RMR (Last-RMR) [20,24,25]. These methods for data selection (i.e. SS or TI) and analysis (i.e. AUC or Last-RMR) significantly impact on RMR or meal-induced thermogenesis estimations [18–21]. However, the impact of the chosen method for data selection and data analysis on the overall measure of CIT and cold-induced nutrient oxidation 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 energy expenditure and nutrient oxidation rates. Despite large scientific interest in cold-induced thermogenesis during the last decade [1,15,26], to our knowledge, there are no studies evaluating the impact of various methodologies on the measurement of cold-induced energy expenditure in healthy human.

MATERIAL AND METHODS

Participants

A total of 63 participants (45 women) participated in the study. The participants were part of the ACTIBATE study, an exercise-based randomized controlled trial (clinicaltrial.gov: NCT02365129) [27]. All participants were young (18-25 years old), healthy, sedentary (<20 min physical activity on <3 days/week), did not smoke or take any medication, had a stable body weight for the past 3 months (<3 Kg change), and were not regularly exposed to cold. The evaluations were performed between October 11th and November 29th, 2016.

The study protocol and informed consent were performed in accordance with the last revision of the Declaration of Helsinki. The study was approved by the Human Research Ethics Committee of the University of Granada (nº924) and of the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada).

Previous conditions to the study days

Participants came to the lab on two separate occasions (5-7 days apart). They were asked to come by bus or by car, under fasting conditions (at least 6 hours), to sleep as usual, to refrain from any moderate (in the previous 24 hours) or vigorous (in the previous 48 hours) physical activity, and not consume alcoholic or stimulant beverages over the past for 6 hours. The participants were evaluated between 8.30 and 19.15hrs. For nutrient oxidation rates analysis, only the participants with a fasting time between 6 and 8 hours were considered [28,29].

Shivering threshold test

During the first study day, we determined the individual shivering threshold. The procedure for the shivering threshold determination has been extensively described elsewhere [30,31]. In brief, participants dressed-up with standardized clothes and stayed seated in a warm room (22.1±1.6ºC) for 30 minutes, before entering the cold (air cooled) room (19.8±0.5ºC), where they were dressed in a temperature-controlled water perfused cooling vest (Polar Products Inc., Ohio, USA) and seated again. The water temperature decreased progressively from 16.6ºC until 3.8ºC or until shivering occurred. Shivering was determined visually and by asking the participants if they were experiencing shivering.

CIT and cold-induced nutrient oxidation rates determination

In the second day, the participants performed the CIT test at the approximate same time of the day at which the shivering threshold test was performed. They dressed-up with the same standardized clothes as in the shivering threshold test. Later, they were moved into the warm room (23.2±0.7ºC). Before being evaluated, all participants lay down on a reclined bed, in a supine position, and were covered by a sheet for 20 minutes. They were instructed to breathe normally, and not to talk, fidget, or sleep. Thereafter, we assessed the RMR maintaining the same standardized conditions [17] during 30 minutes (Figure 1).

After assessing RMR, the participants were moved into the cold room (19.7±0.4ºC) and they put on the temperature-controlled water perfused cooling vest (Polar Products Inc., Ohio, USA) set 4ºC above the individual shivering threshold temperature. Once they had the cooling vest on, they lay down on another reclined bed Indirect calorimetry measurement was performed during two consecutive 30-minute periods, separated by a 5-minute pause to recalibrate the metabolic cart (Figure 1).

The indirect calorimetry measurements for both RMR and CIT were performed with the CCM Express (CCM) or with the Ultima CardiO2 (MGU) (Medgraphics Corp, Minnesota, USA), using a neoprene face-mask equipped with a directconnect™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA) [21]. 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 instructions before every 30 minutes of indirect calorimetry measurement. We used the same metabolic cart for the RMR and CIT measurements in every participant.

Body composition was measured by a DXA scanner (Discovery Wi, Hologic, Inc., Bedford, MA, USA) and data were extracted from the Hologic APEX 4.0.2. (Hologic, Inc., Bedford, MA, USA), and weight and height were measured with a Seca scale and a stadiometer (model 799, Electronic Column Scale, Hamburg, Germany).

Methods for data selection and analysis

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 coefficients of variance for oxygen consumption, carbon dioxide production, minute ventilation, and respiratory exchange ratio (RER)) [21].

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 indirect calorimetry instrument. After excluding the first 5 minutes of every 30-minute record [18], we used three different methods for data selection (Figure 1): i) TI every 5 minutes (*5min-TI*): mean values of every consecutive 5-minute period (i.e. from the 6^{th} to the 10^{th},

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

197 In order to express the CIT as a single value, we used two different methods for data
198 analysis (Figure 1): i) The AUC following the trapezoidal rule; and ii) the Last-RMR.
199 Both methods for data analysis were expressed as a percentage of the baseline RMR.

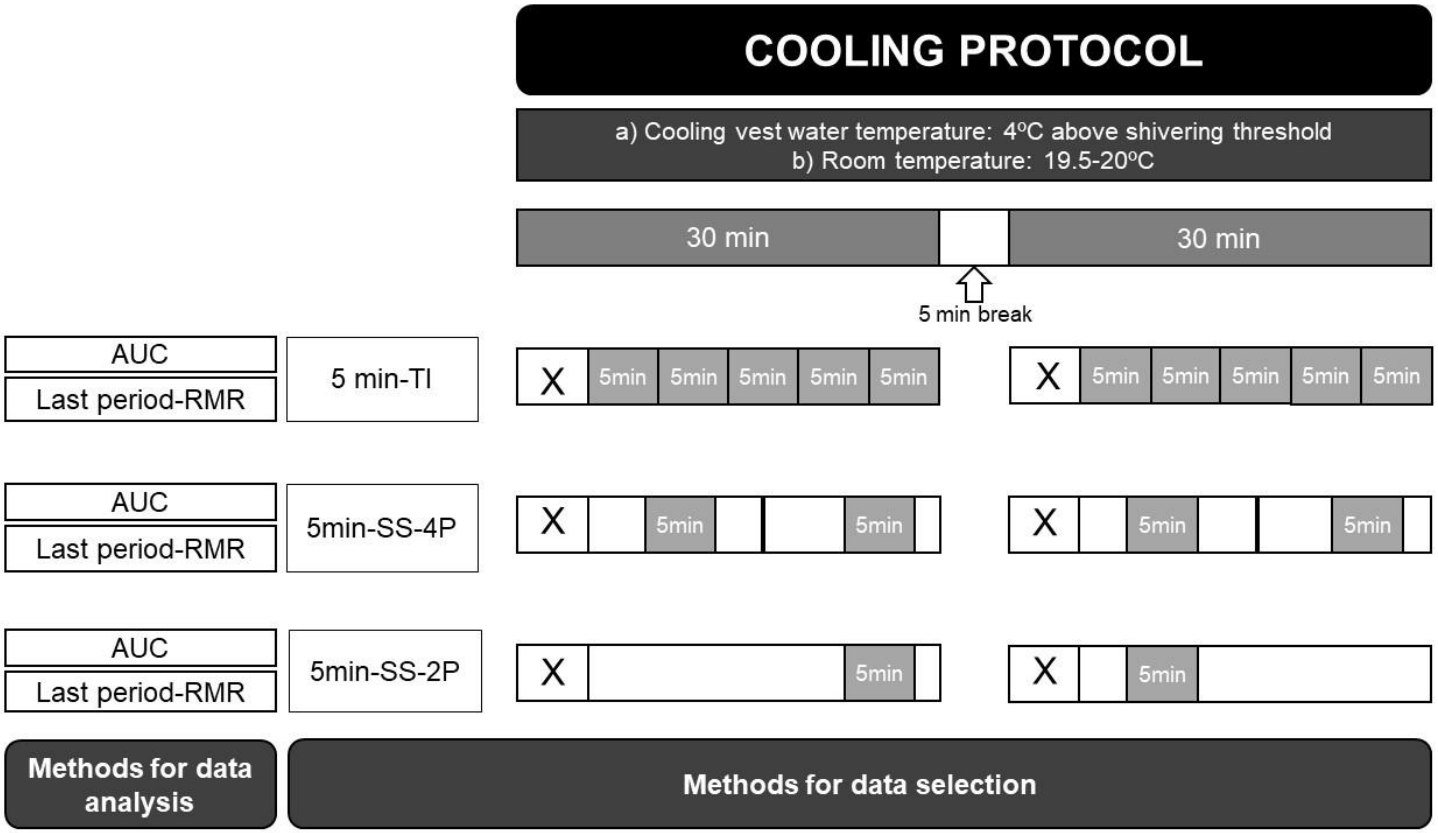


Figure 1. Cooling protocol, methods for data selection, and methods for data analysis. White rectangles in cooling protocol represent every 30 minutes of recorded gas exchange. Gray squares represent the 5-minute selected period within a specific recorded time (i.e. in the time interval method: average 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.

Oxygen consumption and carbon dioxide production for each selected data point were used to estimate EE, and carbohydrates (CHOox) and fat oxidation (FATox). EE was estimated through Weir´s abbreviated equation, not considering urinary nitrogen concentration [32]. For carbohydrates and fat oxidation estimations, we used Frayn´s equation, not considering urinary nitrogen concentration [33].

Statistical analysis

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 at <0.05.

A repeated-measures analysis of variance (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.

RESULTS

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) [17]. Finally, a total of 44 participants were included in the energy expenditure analysis (Table 1). 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 analysis (Table S1).

Table 1. 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; VO_2: resting oxygen consumption; VCO_2: resting carbon dioxide production; RMR: Resting Metabolic Rate; RER: resting respiratory exchange ratio.

Cold-induced thermogenesis

Figure 2 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.

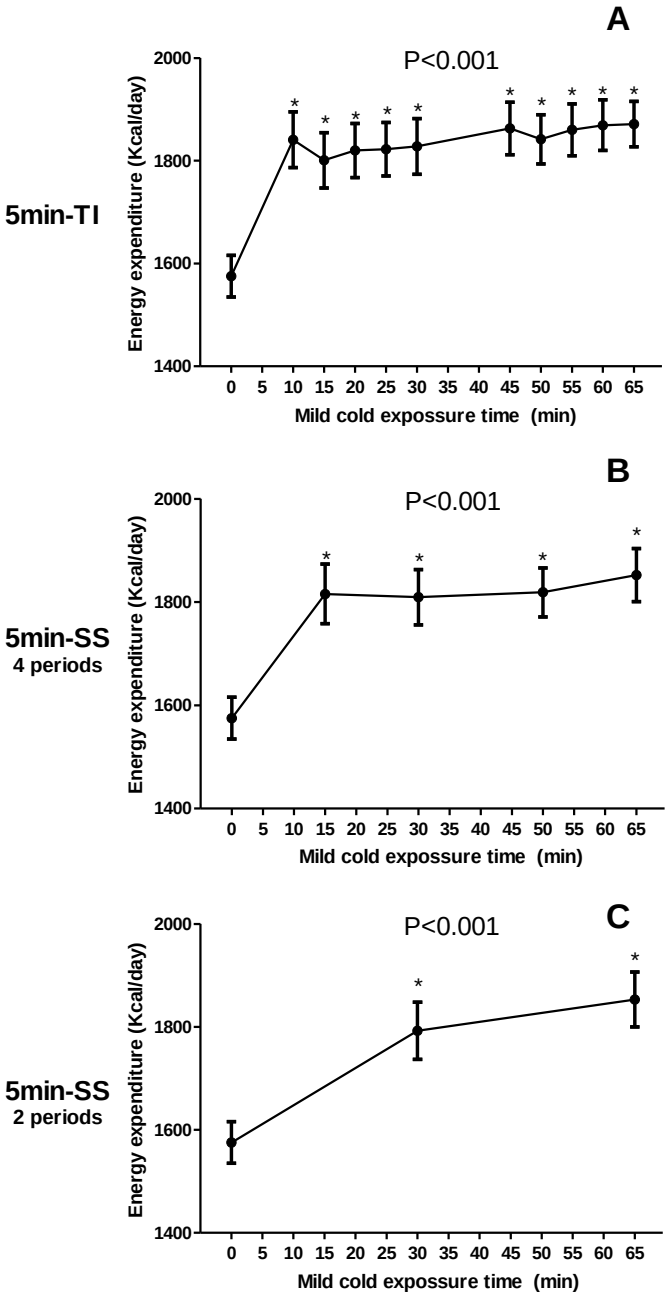


Figure 2. Energy expenditure (EE) during mild cold exposure by methods for data selection. Represented values are mean ± standard error. Panel A presents data obtained with the 5-minute time interval (TI) method; Panel B presents data obtained with the steady state (SS) method after dividing the cold 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.

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 S1 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 3 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 $P\leq0.043$). 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 ($P<0.001$).

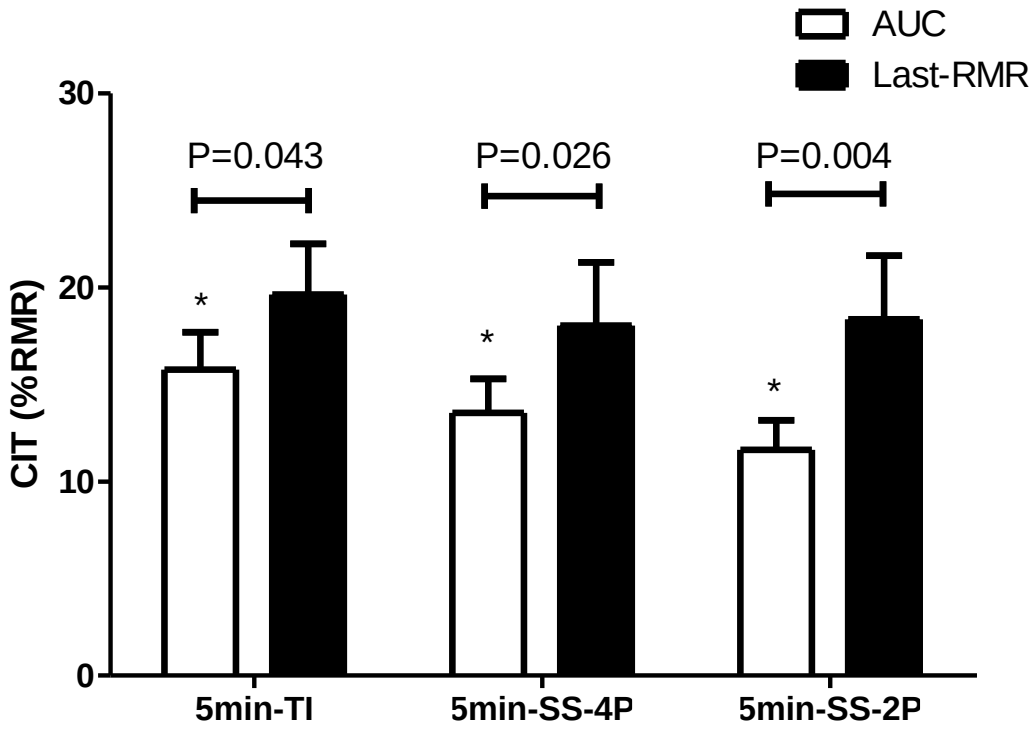
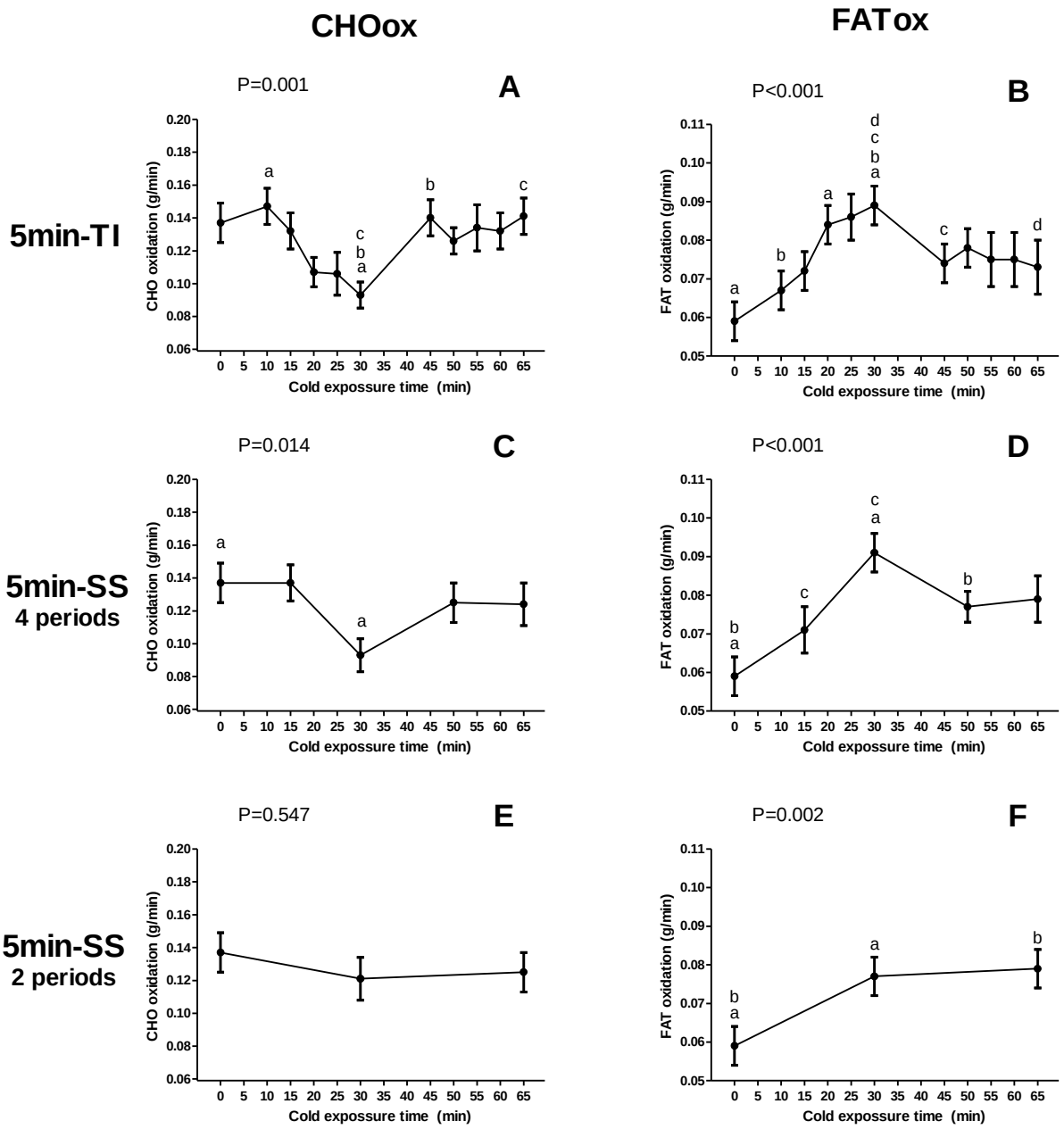


Figure 3. Comparisons between mean over-all cold-induced thermogenesis (CIT) obtained with different methods for data selection and analysis. Represented values are mean ± standard error. P values for paired *t*-test. *: significant differences with the rest of the AUC results after Bonferroni correction. 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.

Cold-induced nutrient oxidation rates

Figure 4 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*.



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290 **Figure 4.** Nutrient oxidation rates during cold exposure by method for data selection.
291 Panels A and B present data obtained with the 5-minute time interval (TI) method; panels
292 C and D present data obtained with the steady state (SS) method after dividing the cold
293 exposure into 4 periods; and panels E and F present data obtained with the steady state
294 method after dividing the cold exposure into 2 periods. Represented values are mean ±
295 standard error. P value for repeated measures ANOVA. Equal lower-case letters indicate
296 significant differences after Bonferroni correction. CHO: Carbohydrates. min: minutes;
297 g: grams.

298 Regarding mean overall cold-induced nutrient oxidation rates estimation, no differences
299 were found when comparing the methods for data selection (P=0.181), nor when
300 comparing the methods for data analysis (P=0.328) (Figure S2).

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 energy expenditure and nutrient oxidation rates during a 60-minute individualized mild cold exposure, designed to elicit maximum non-shivering thermogenesis. 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 cold-induced nutrient oxidation rates. 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 energy expenditure during cold exposure.

The selection of the method for data selection and analysis influences the estimations of RMR and meal-induced thermogenesis [17–22]. Therefore, it is expected that the selection of the method for data selection and analysis also influences the estimation of CIT and cold-induced nutrient oxidation rates. 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 4). 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 [19,21,22]. 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 S2A), as previously argued [19,21,22]. 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 S1). 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 cold-induced nutrient oxidation rates, as it allows a more detailed insight (Figure 2 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 S1). 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 3). This could be explained by the fact that energy expenditure 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 S1) 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 [26,34,35]. 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. [26]. 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 [17–19,21].

Limitations

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 non-shivering thermogenesis, we cannot be sure of the relative contribution to CIT of shivering thermogenesis [12]. 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 non-shivering thermogenesis is predominant in the included participants. Thirdly, we used two different metabolic carts which are not comparable and have relatively low reliability [36–38]. 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.

Conclusions

The methods for data selection and analysis can have a profound impact on CIT and cold-induced nutrient oxidation rates 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 cold-induced nutrient oxidation rates, while *5min-SS-4P* and AUC should be the method of choice when computing CIT and cold-induced nutrient oxidation rates as a single value.

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AUTHOR´S CONTRIBUTION

GSD, JMA, and JRR conceived the study; GSD, JMA, ML, IL and JRR designed the study; GSD, JMA, FMA, BMT, FAG and LOA did the data collection; GSD performed the statistical analyses and drafted the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST SOURCES

The authors confirm that there are no conflicts of interest.

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516

SUPPLEMENTARY MATERIAL

Table S1. Descriptive characteristics of the participants included in the nutrient oxidation rate analysis.

	All (n=18)		Male (n=5)		Female (n=13)	
Age (years)	21.9	(2.0)	21.6	(2.2)	22.0	(2.0)
BMI (kg/m^2)	24.3	(4.6)	26.5	(5.3)	23.5	(4.1)
Lean mass (kg)	40.4	(8.0)	50.7	(5.7)	36.5	(4.5)
Fat mass (kg)	25.0	(9.6)	28.4	(13.0)	23.7	(8.2)
Fat mass (%)	36.1	(7.0)	33.5	(7.3)	37.1	(6.9)
VO_2 (ml/min)	222	(33)	248	(43)	211	(22)
VCO_2 (ml/min)	186	(27)	206	(31)	178	(23)
RMR (kcal/day)	1554	(226)	1738	(292)	1483	(157)
RER	0.842	(0.048)	0.835	(0.025)	0.846	(0.055)

Data are presented as means (standard deviation). BMI: Body mass index; VO_2: resting oxygen consumption; VCO_2: resting carbon dioxide production; RMR: Resting Metabolic Rate; RER: resting respiratory exchange ratio.

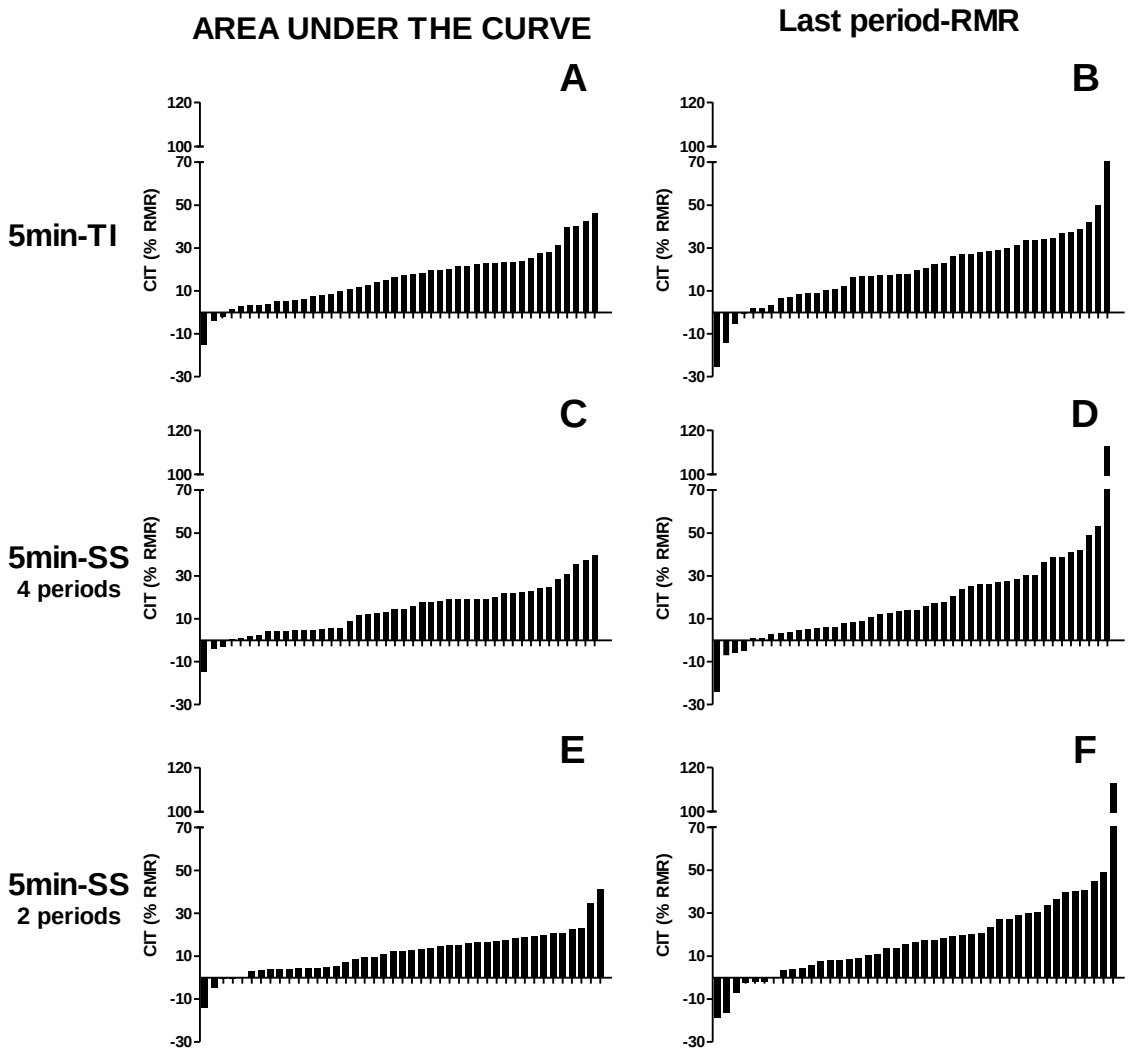

Figure S1. Individual data for over-all cold-induced thermogenesis (CIT) obtained with different methods for data selection and analysis. Panels A and B represent data obtained with the 5-minute time interval (TI) method for data selection. Panels C and D represent data obtained with the steady state (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.

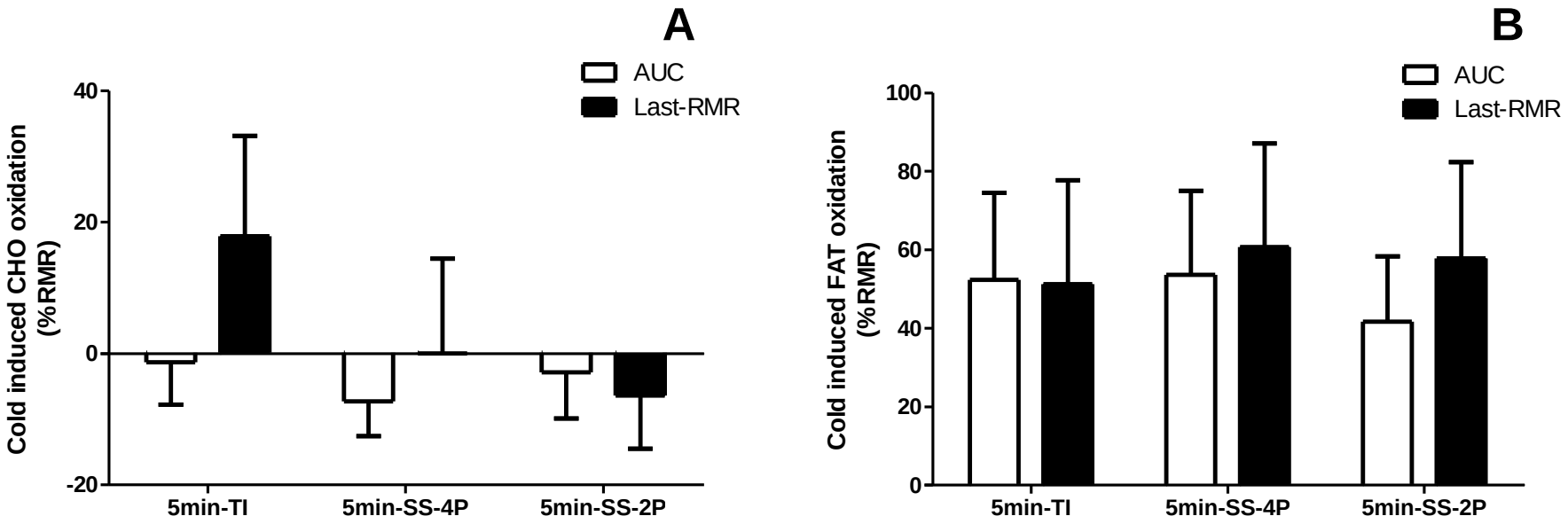

Figure S2. Comparisons between mean over-all cold-induced nutrient (carbohydrates (CHO) and fat (FAT)) oxidation rates obtained with different methods for data selection and analysis. TI: Time interval; SS: Steady State; RMR: Resting metabolic rate; AUC: Area under the curve; Last-RMR: Last period value 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.