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# Resistance to lean mass gain in constitutional thinness in free-living conditions is not overpassed by overfeeding

Yiin Ling<sup>1,2</sup>, Bogdan Galusca<sup>1,2</sup> , François-Pierre Martin<sup>3</sup>, Simona Bartova<sup>3</sup>, Jérôme Carayol<sup>3</sup>, Sofia Moco<sup>3</sup>, Jacques Epelbaum<sup>4</sup>, Dominique Grouselle<sup>4</sup>, Yves Boirie<sup>5</sup>, Christophe Montaurier<sup>5</sup>, Joyceline Cuenco<sup>6</sup>, James S. Minnion<sup>6</sup>, Thierry Thomas<sup>7</sup>, Sylvie Mure<sup>1</sup>, Jörg Hager<sup>3</sup>, Bruno Estour<sup>1,2</sup>, Nele Gheldof<sup>3</sup> & Natacha Germain<sup>1,2\*</sup> 

<sup>1</sup>Division of Endocrinology, CHU Saint-Etienne, Saint-Etienne, France, <sup>2</sup>Eating Disorders, Addictions & Extreme Bodyweight Research Group, Jean Monnet University, Saint-Etienne, France, <sup>3</sup>Metabolic Health, Nestlé Research, EPFL Innovation Park, Lausanne, Switzerland, <sup>4</sup>Psychiatry and Neurosciences Center, Paris Descartes University, INSERM UMR 894, Paris, France, <sup>5</sup>Human Nutrition Unit, INRA, Research Center, UMR 1019, Clermont-Ferrand, France, <sup>6</sup>Division of Diabetes, Endocrinology and Metabolism, Imperial College, London, UK, <sup>7</sup>Rheumatology Department, CHU Saint-Etienne, Saint-Etienne, France

## Abstract

**Background** Constitutional thinness (CT), a non-malnourished underweight state with no eating disorders, is characterized by weight gain resistance to high fat diet. Data issued from muscle biopsies suggested blunted anabolic mechanisms in free-living state. Weight and metabolic responses to protein caloric supplementation has not been yet explored in CT.

**Methods** A 2 week overfeeding (additional 600 kcal, 30 g protein, 72 g carbohydrate, and 21 g fat) was performed to compare two groups of CTs (12 women and 11 men) to normal-weight controls (12 women and 10 men). Bodyweight, food intake, energy expenditure, body composition, nitrogen balance, appetite hormones profiles, and urine metabolome were monitored before and after overfeeding.

**Results** Before overfeeding, positive energy gap was found in both CT genders (309 ± 370 kcal in CT-F and 332 ± 709 kcal in CT-M) associated with higher relative protein intake per kilo (1.74 ± 0.32 g/kg/day in CT-F vs. 1.16 ± 0.23 in C-F,  $P < 0.0001$ ; 1.56 ± 0.36 in CT-M vs. 1.22 ± 0.32 in C-M,  $P = 0.03$ ), lower nitrogen (7.26 ± 2.36 g/day in CT-F vs. 11.41 ± 3.64 in C-F,  $P = 0.003$ ; 9.70 ± 3.85 in CT-M vs. 14.14 ± 4.19 in C-M,  $P = 0.02$ ), but higher essential amino acids urinary excretion (CT/C fold change of 1.13 for leucine and 1.14 for arginine) in free-living conditions. After overfeeding, CTs presented an accentuated positive energy gap, still higher than in controls (675 ± 540 in CTs vs. 379 ± 427 in C,  $P = 0.04$ ). Increase in lean mass was induced in both controls genders but not in CTs (a trend was noticed in CT women), despite a similar nitrogen balance after overfeeding (5.06 ± 4.33 g/day in CTs vs. 4.28 ± 3.15 in controls,  $P = 0.49$ ). Higher anorectic gut hormones' tone, glucagon-like peptide 1 and peptide tyrosine tyrosine, during test meal and higher snacking frequency were noticed before and after overfeeding in CTs.

**Conclusions** The blunted muscle energy mechanism, previously described in CTs in free-living state, is associated with basal saturated protein turn over suggested by the concordance of positive nitrogen balance and an increased urine excretion of several essential amino acids. This saturation cannot be overpassed by increasing this spontaneous high-protein intake suggesting a resistance to lean mass gain in CT phenotype.

**Keywords** Overfeeding; Constitutional Thinness; Bodyweight gain; Energy gap; Nitrogen balance

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\*Correspondence to: Natacha Germain, MD, PhD, Endocrinology Department, University Hospital of Saint-Etienne, 42055 Saint-Etienne Cedex 2, France. Phone: +33 4 77 12 77 27; Fax: +33 4 77 12 04 93. Email: natacha.germain@chu-st-etienne.fr

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Ethical approval has been obtained from Ethics Committee France: ANSM (2013-A00590-45).

This study is registered in Clinical Trial.gov, no. NCT02004821 (<https://clinicaltrials.gov/ct2/show/NCT02004821?term=thinness&rank=3>).

## Background

Bodyweight maintenance is related to the balance between energy intake and expenditure, with interindividual physiological variations, from thinness to obesity.<sup>1</sup> A recent cohort publication showed the difference of naturally underweight women from anorexia nervosa.<sup>2</sup> Indeed, this low bodyweight condition [body mass index (BMI) < 17.5 kg/m<sup>2</sup>] also called constitutional thinness (CT) is a state without any sign of undernutrition as observed through normal nutritional biomarkers and gonadal function and low but not blunted leptin plasma levels.<sup>1–5</sup> CT also present with less psychological dietary restriction behaviours compared with normal-weight women.<sup>2</sup> CT individuals have a steady bodyweight in the lower population-wide percentiles adjusted for age, gender, and ethnicity,<sup>2,3</sup> suggestive of a genetic determination of this underweight state consistent with previous reports on the heritability of thinness.<sup>6–11</sup> The CT condition also impacts one's quality of life, CT patients being generally unsatisfied by their low body weight and inability to gain weight and often consult for medical advice.<sup>2,12</sup>

A more mechanistic understanding of this phenotype is currently lacking yet would be important to identify potential therapeutic targets. As a proof of concept, a recent 4 week fat overfeeding study, an excess of more than 600 kcal per day in fat, revealed a form of resistance to bodyweight and fat mass gain in a group of CT women, and that despite an enhanced paradoxical positive energy gap (higher food consumed than energy expended),<sup>13</sup> This could reflect the paradoxical negative energy gap suggested in genetic obesity.<sup>14</sup> Although this gap may be interpreted as a food intake reporting issue, hypothesis in energy pathways specific to CT population should not be neglected. Recently, we have shown the CT condition is also marked by a distinct skeletal muscle phenotype, as energy storage defects were observed in muscle biopsies that may partly contribute to body weight gain resistance.<sup>15</sup> Yet molecular processes behind protein storage and turn over are poorly understood in this population.

In the present study, we aimed at further studying the protein metabolism of CT male and female subjects compared with control individuals. We conducted an overfeeding intervention in both groups using Renutryl® Booster to provide additional 600 kcal, 30 g protein, 72 g carbohydrate, and 21 g fat intake per day for 2 weeks. Using a combination of anthropometric, clinical, and metabolic phenotyping measures, we explored clinical end points related to energy and protein balance, appetite regulation, and 24 h urinary metabolomics.<sup>16</sup>

## Methods

### Ethics

This clinical investigation was conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments (as revised in 1983). Study was approved by the local research and ethics committee of Saint-Etienne, France, ANSM (2013-A00590-45) and registered at clinicaltrial.gov as NCT02004821. All subjects gave a written informed consent.

### Subjects

A total of 67 healthy young subjects were recruited, of which a total of 60 completed the study protocol (dropouts: one female CT, three male CTs, two female controls, one male control): 15 female CT (CT-F), 15 male CT (CT-M), 15 female controls (C-F), and 15 male controls (C-M) (see Supporting information, **Supplementary figure 1**).

CT subjects (BMI < 18.5 kg/m<sup>2</sup>) were recruited amongst our outpatients all of whom wished to gain weight, had stable bodyweight throughout post-pubertal period confirmed by their personal weight history, without eating disorders as evaluated by the Eating Disorder Examination Questionnaire and Dutch Eating Behavior Questionnaire, and no amenorrhoea for women after hormonal contraception withdrawal when relevant.<sup>2</sup> CT non-undernourished state of underweight was supported by normal nutritional biomarkers including normal levels of insulin-like growth factor 1 (IGF-1) and free triiodothyronine (FT<sub>3</sub>).<sup>2</sup> They displayed no hepatic disorders and no over exercise behaviour according to the MONICA Optional Study of Physical Activity Questionnaire.<sup>17</sup>

Normal-weight subjects (BMI 20–25 kg/m<sup>2</sup>) without any eating disorders and any medication were recruited by advertising to serve as control group.

All subjects were recruited within an age range between 18 and 36 years, had a stable body weight for at least 3 months, and did not take any medication. All criteria of inclusion and exclusion were previously fully detailed.<sup>16</sup>

### Study design and dietary intervention

After 2 week of baseline assessments (day D1–D11, visits V1–V4), all participants were asked to consume an extra bottle of Renutryl® Booster [72 g carbohydrates (48.5%), 30 g proteins (20%), and 21 g fat (31.5%)] as an add-on to their usual food intake during 14 consecutive days (D11–D25, V5–V8).

Participants were required to consume the bottle in the interval of dinner-to-bed time in free-living conditions and to maintain their usual dietary and normal lifestyle throughout the study. Bodyweight was measured throughout the study and was followed-up at 2 week post-overfeeding (ad libitum free-living period) (D43, V9). Scheduled appointments allowed to regularly check the compliance in order to avoid compensatory behaviours.<sup>18</sup> Study protocol design was previously extensively described in a specific design article.<sup>16</sup>

### *Anthropometric and body composition measurements*

All subjects underwent anthropometric measurements in the early morning at fasting state at each visit. Total body weight (TBW) was measured with 0.1 kg precision and height with 0.1 cm. Body composition was assessed, at baseline and at post-overfeeding, to determine the distribution of total body fat mass (FM) and fat-free mass using dual-energy X-ray absorptiometry.<sup>19</sup>

### *Energy balance assessments*

Energy balance assessments were performed before and after overfeeding both under free-living and experimental conditions.

#### *Under free-living conditions*

Resting energy expenditure (REE) was measured at 12 h fasting state in supine position by indirect calorimetry using a canopy (Quark RMR, COSMED, Italy).<sup>20</sup> Accelerometer (Actiheart, CamNtech, Cambridge, UK)<sup>21</sup> was used to monitor physical activity level (PAL) in real life. Free-living total energy expenditure (TEE) was obtained from calculation:  $TEE = REE \times PAL$ . Fat and carbohydrate oxidation was assessed using Ferrannini's equations.<sup>22</sup>

Usual caloric consumption and eating habits were assessed using a daily self-reporting dietary record during 7 days, with the guide of a photographic reference book previously validated.<sup>23</sup>

Dietary record was checked by a dietician to ensure accuracy of data collection. Total daily energy intake (TEI) was calculated using GENI software (MICRO6, France) and the French Food and Nutrient Files CNEVA-CIQUAL.<sup>24</sup> Snacking was defined as food intake out of the 3 main meals (breakfast, lunch and dinner). The ratio of TEI/REE was used to detect misreporting of dietary intake, with underreporting  $<1.35$  and overreporting  $>2.10$ .<sup>25</sup>

#### *Under experimental conditions*

Experimental TEE was measured using a whole-body calorimetric chamber, an open-circuit indirect calorimetric system (HNRC, Auvergne, France), previously described.<sup>26</sup> Volunteers

spent 38 h in calorimetric chambers. They entered at 5 p.m. the first evening for an adaptation night, and the experiment started at 7 a.m. the next time, and ended 24 h later. Identical standardized controlled meals consisting of 2300 kcal per day (three main and one snacking in the afternoon) were given to participants, enough to cover their usual food intake according to ambulatory evaluation. No extra outside food was allowed. Real food intake was measured by dietitians in order to be accurate with simultaneous energy expenditure measurements.

### *Samplings*

Baseline venous samples were collected after a 12 h overnight fasting for the measurement of serum leptin, albumin, free T3, IGF-1, insulin, blood glucose, triglycerides, non-esterified fatty acids, and glycerol. Homeostatic Model assessment of insulin resistance was calculated according to the formula:  $\text{fasting insulin } (\mu\text{UI/L}) \times \text{fasting glucose } (\text{mmol/L})/22.5$ .<sup>27</sup>

Twenty-four hour urine samples were collected for nitrogen loss determination as well as metabolome analysis at different visits, including baseline visits (V2 and V3) and visits during overfeeding (V6, V7, and V8).

Standardized test meals were performed before (V2), and at the end of the 2 week overfeeding period (V8), using a bottle of Renutryl® Booster, consumed slowly during 15 min under surveillance. Venous blood samples were collected in tubes containing aprotinin and EDTA at 7 time points:  $T_0 = 0$  min (after a 12 h overnight fasting),  $T_{15} = 15$  min, (immediately after Renutryl® Booster consumption),  $T_{30} = 30$  min,  $T_{60} = 60$  min,  $T_{90} = 90$  min,  $T_{120} = 120$  min, and  $T_{150} = 150$  min, to assay blood glucose, insulin, total ghrelin and acyl-ghrelin (AG), peptide tyrosine tyrosine (PYY), and glucagon-like peptide 1 (GLP-1) concentrations. Samples were immediately centrifuged at  $+4^\circ\text{C}$ , aliquoted, and kept frozen at  $-20^\circ\text{C}$  before stocked at  $-80^\circ\text{C}$  until assays. A 1 N HCl was added to the final concentration of 0.1 HCl into an aliquot dedicated to ghrelin assay in order to enhance acylated ghrelin stability.

### *Assays*

Standardized techniques for assessment of plasma parameters were previously described.<sup>16</sup> Urinary urea/24 h was measured based on enzymatic reaction with urease and glutamate dehydrogenase. Nitrogen (N) intake was assessed with self-reported diet diaries (free living) and controlled weighed food by dietician (calorimetric chamber), with 6.25 g of protein per g of N. N losses were calculated based on Lee and Hartley formula:  $\text{nitrogen excretion (g N/24 h)} = \text{urinary urea (mmol/24 h)} \times 0.028 \times 1.2$  (factor of non-urea urine N losses).

## Urinary metabolomics

Following homogenization, aliquots of 24 h urine samples were prepared and kept frozen at  $-80^{\circ}\text{C}$  until analysis. Metabolomics analysis was carried out by  $^1\text{H}$  NMR spectroscopy in-house at NIHS, Switzerland, using established procedures.<sup>28</sup> Exhaustive description of the method is presented in the supplementary method. A total of 79 signals out of 155 were assigned to biochemical molecular species, corresponding to 51 unique metabolites. The signals were expressed in arbitrary units corresponding to peak area normalized to total spectral area or creatinine peak area. This approach allowed for the detection of major metabolic intermediates belonging to central metabolism, including amino acids, organic acids, and sugars, as well as aromatic-containing compounds.

## Statistical analysis

In the current study, only participants with complete data and who really complied with the overfeeding were included in statistical analyses. Compliance was defined using the following criteria: increased food intake above 450 kcal per day,<sup>13</sup> positive change in urine urea,<sup>29</sup> and no increase in PAL during the overfeeding period. Intergroup differences and the effects of the short-term overfeeding within each group were evaluated in 12 female CTs (CT-F), 11 male CTs (CT-M), 12 female controls (C-F), and 10 male controls (C-M).

All data are presented as mean  $\pm$  SD. Homogeneity of data was checked with the Kolmogorov–Smirnov test, and transformation was applied when data were not normally distributed.

Mann–Whitney’s non-parametric unpaired test was used to compare one-time measured parameters (including meal test mean values) between CT and controls groups (including both genders and for each gender separately) at each visit. Wilcoxon signed rank’s non-parametric tests were used to analyse the differences before vs. after overfeeding points for a given parameter within each group (including both genders and for each gender separately). Correlations between every baseline parameters and basal gap were also evaluated in order to find out potential predictive markers of the energy gap. Statistical significance was set at  $P < 0.05$ .

A one-factor (time) repeated measures analysis of variance was used to analyse the parameters changes over the visits and appetite-regulatory hormones changes during each test meal in each group (including both genders and for each gender separately). Fisher’s PLSD post hoc tests between two assessment points were performed when time effect was significant ( $P < 0.05$ ).

Multiple testing was taken into account by correcting the  $P$  values using the Bonferroni method.

Statistical analyses and graphs were performed with StatView 4.5 (Abacus Concepts, Inc. CA) and GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

Regarding the urinary metabolomics data, initial data analyses were conducted using principal component analysis in order to assess metabolic similarities between samples and outlier detection. Further characterization of discriminating metabolite phenotype was performed using supervised multivariate data analysis of total area and creatinine normalized data, using orthogonal projection to latent structures discriminant analysis (orthogonal partial least squares discriminant analysis,<sup>30</sup>). To analyse metabolites longitudinal information, we applied an analysis of variance for repeated measurements with age and sex as confounders and CT status as main effect. An interaction term between visit and status parameters was added to the model to assess a potential different metabolite trajectory between the two groups. All metabolites were log2 transformed before analysis. The same analysis was also performed after a rank transformation to normality of the metabolites values to check for false positive results because of an excess of outliers or an excess of distribution skewness. Correction for multiple testing was applied using the Benjamini–Hochberg method.

## Results

### Baseline characteristics

Constitutional thinness subjects in both genders had lower mean body weight, BMI, fat mass (absolute and relative values), lean mass (absolute value), and leptin levels, as compared with control subjects. However, there were no differences between CTs and controls in nutritional markers, including albumin, triglycerides, non-esterified fatty acids, and glycerol, free  $\text{T}_3$ , and IGF-1. All subjects were insulin-sensitive displaying normal glucose tolerance (*Table 1*).

Free-living TEI in kcal was slightly higher in female CTs compared with female controls and was similar between CTs and male controls. Daily snacking calorie contribution was significantly greater in CTs compared with controls (mean  $16.76 \pm 10.31\%$  vs.  $10.55 \pm 8.65\%$ , respectively,  $P = 0.0142$ ). Baseline food-reporting mean ratio was calculated at  $1.325 \pm 0.052$  in female controls;  $1.937 \pm 0.113$  in female CTs;  $1.482 \pm 0.057$  in male controls, and  $1.684 \pm 0.092$  in male CTs (*Table 2*).

Absolute protein intake was similar between CTs and controls in both genders (*Table 3*). However, when reported to bodyweight as proposed in protein dietary reference intakes, CTs’ protein intake was significantly higher compared with controls in both genders (*Table 3*). Absolute nitrogen losses were significant lower in both female and male CTs, and

**Table 1** Baseline clinical characteristics of all participants

| Parameters                     | CT-F         | C-F          | P value (females) | CT-M         | C-M          | P value (males) |
|--------------------------------|--------------|--------------|-------------------|--------------|--------------|-----------------|
| <b>Body composition</b>        |              |              |                   |              |              |                 |
| Age (years)                    | 26.9 ± 4.7   | 21.9 ± 3.0   | 0.0018            | 23.0 ± 3.9   | 23.3 ± 2.8   | 0.7889          |
| BMI (kg/m <sup>2</sup> )       | 16.57 ± 0.72 | 22.88 ± 1.17 | <0.0001           | 17.31 ± 0.73 | 22.94 ± 0.99 | <0.0001         |
| Weight (kg)                    | 42.85 ± 4.44 | 62.26 ± 4.72 | <0.0001           | 53.57 ± 2.73 | 74.86 ± 7.08 | <0.0001         |
| <b>Nutritional biomarkers</b>  |              |              |                   |              |              |                 |
| Leptin (ng/mL)                 | 4.53 ± 1.85  | 13.29 ± 6.22 | <0.0001           | 0.95 ± 0.09  | 3.30 ± 2.80  | 0.0031          |
| Free T3 (pmol/L)               | 6.2 ± 2.7    | 5.4 ± 0.6    | 0.2999            | 5.6 ± 0.6    | 5.3 ± 0.8    | 0.3970          |
| IGF-1 (µg/L)                   | 239 ± 17     | 274 ± 10     | 0.0893            | 221 ± 29     | 255 ± 21     | 0.3938          |
| Albumine (g/L)                 | 0.26 ± 0.06  | 0.28 ± 0.04  | 0.5408            | 0.30 ± 0.04  | 0.30 ± 0.05  | 0.9906          |
| <b>Metabolic parameters</b>    |              |              |                   |              |              |                 |
| Fasting insulin (UI/L)         | 5.68 ± 2.33  | 7.31 ± 2.18  | 0.0629            | 6.43 ± 4.61  | 7.41 ± 3.39  | 0.5122          |
| Fasting blood glucose (nmol/L) | 4.61 ± 0.41  | 4.61 ± 0.50  | 0.9999            | 4.67 ± 0.28  | 4.71 ± 0.38  | 0.7844          |
| HOMA-IR                        | 1.18 ± 0.53  | 1.51 ± 0.53  | 0.1061            | 1.35 ± 1.00  | 1.57 ± 0.75  | 0.5008          |
| TG (mmol/L)                    | 0.96 ± 0.27  | 0.82 ± 0.33  | 0.2348            | 0.90 ± 0.25  | 0.91 ± 0.25  | 0.8699          |
| NEFA (µmol/L)                  | 459 ± 236    | 477 ± 239    | 0.8335            | 313 ± 126    | 251.6 ± 140  | 0.2111          |
| Glycerol (µmol/L)              | 45.4 ± 31.4  | 34.1 ± 22.1  | 0.2874            | 15.8 ± 13.8  | 14.1 ± 11.8  | 0.7148          |

Mean ± SD. Statistical significance when *P* value <0.05.

BMI, body mass index; C, controls; CT, constitutional thinness; F, female; HOMA-IR, homeostatic model assessment for insulin resistance; NEFA, non-esterified fatty acids; M, male; TG, triglycerides.

**Table 2** Energetic and metabolic parameters for all groups at baseline and at post-overfeeding

| Parameters  |        | Baseline    |             |                | Post-overfeeding |             |                | Time<br><i>P</i> value<br>(CTs) | Time<br><i>P</i> value<br>(controls) |
|---|--------|-------------|-------------|----------------|------------------|-------------|----------------|---------------------------------|--------------------------------------|
|   |        | CTs         | Controls    | <i>P</i> value | CTs              | Controls    | <i>P</i> value |                                 |                                      |
| Fat mass (kg)   | Female | 10.3 ± 1.5  | 19.7 ± 2.9  | <0.0001        | 10.5 ± 1.3       | 19.9 ± 2.7  | <0.0001        | 0.1485                          | 0.0500                               |
|   | Male   | 8.2 ± 1.2   | 17.1 ± 6.8  | <0.0001        | 8.6 ± 1.5        | 17.3 ± 6.7  | <0.0001        | 0.0183                          | 0.0566                               |
|   | Total  | 9.3 ± 1.7   | 18.5 ± 5.1  | <0.0001        | 9.6 ± 1.7        | 18.7 ± 5.0  | <0.0001        | 0.0051                          | 0.0050                               |
| Lean mass (kg)  | Female | 32.4 ± 2.7  | 42.4 ± 3.2  | <0.0001        | 32.9 ± 3.1       | 43.3 ± 2.9  | <0.0001        | 0.0733                          | 0.0011                               |
|   | Male   | 45.4 ± 2.5  | 58.1 ± 6.5  | <0.0001        | 45.6 ± 2.2       | 59.0 ± 6.9  | <0.0001        | 0.4425                          | 0.0067                               |
|   | Total  | 38.9 ± 7.1  | 49.5 ± 9.4  | <0.0001        | 39.3 ± 7.0       | 50.4 ± 9.4  | <0.0001        | 0.0665                          | <0.0001                              |
| Resting energy expenditure (kcal/24 h)                    | Female | 1059 ± 129  | 1344 ± 103  | 0.0007         | 1119 ± 122       | 1377 ± 124  | <0.0001        | 0.0021                          | 0.3325                               |
|   | Male   | 1444 ± 130  | 1590 ± 280  | 0.0005         | 1398 ± 191       | 1762 ± 238  | <0.0001        | 0.7588                          | 0.0090                               |
|   | Total  | 1252 ± 233  | 1456 ± 234  | <0.0001        | 1258 ± 212       | 1552 ± 266  | <0.0001        | 0.1302                          | 0.0075                               |
| Free living Total energy intake (kcal/24 h)               | Female | 2039 ± 306  | 1788 ± 303  | 0.0144         | 2553 ± 254       | 2429 ± 240  | 0.2306         | 0.0002                          | <0.0001                              |
|   | Male   | 2425 ± 534  | 2349 ± 583  | 0.9971         | 2618 ± 430       | 2986 ± 514  | 0.1035         | 0.1459                          | 0.0010                               |
|   | Total  | 2232 ± 469  | 2043 ± 525  | 0.2043         | 2586 ± 386       | 2681 ± 473  | 0.4526         | 0.0003                          | <0.0001                              |
| Free living Total energy expenditure (kcal/24 h)          | Female | 1730 ± 266* | 1989 ± 325* | 0.0439         | 1792 ± 275*      | 2011 ± 328* | 0.0901         | 0.2794                          | 0.8168                               |
|   | Male   | 2093 ± 429* | 2506 ± 607  | 0.0935         | 2029 ± 539*      | 2651 ± 588* | 0.0176         | 0.6298                          | 0.0949                               |
|   | Total  | 1912 ± 429* | 2224 ± 532  | 0.0330         | 1911 ± 436*      | 2302 ± 558* | 0.0107         | 0.7520                          | 0.1048                               |
| Calorimetric chamber Total energy intake (kcal/24 h)      | Female | 1903 ± 215  | 2022 ± 169  | 0.1464         | 2464 ± 225       | 2635 ± 191  | 0.0563         | <0.0001                         | <0.0001                              |
|   | Male   | 2082 ± 173  | 2166 ± 152  | 0.2461         | 2583 ± 290       | 2738 ± 155  | 0.1461         | <0.0001                         | <0.0001                              |
|   | Total  | 1993 ± 212  | 2088 ± 174  | 0.1056         | 2523 ± 261       | 2682 ± 179  | 0.0217         | <0.0001                         | <0.0001                              |
| Calorimetric chamber Total energy expenditure (kcal/24 h) | Female | 1616 ± 186* | 2003 ± 187  | <0.0001        | 1607 ± 2011*     | 2141 ± 167* | <0.0001        | 0.9754                          | 0.0004                               |
|   | Male   | 1998 ± 138  | 2398 ± 252  | <0.0001        | 2063 ± 146*      | 2498 ± 206* | <0.0001        | 0.0666                          | 0.0167                               |
|   | Total  | 1807 ± 252* | 2182 ± 293  | <0.0001        | 1835 ± 293*      | 2303 ± 257* | <0.0001        | 0.2643                          | <0.0001                              |
| Fat oxidation rate (mg/min/kg)                            | Female | 1.2 ± 0.4   | 1.3 ± 0.4   | 0.7031         | 0.8 ± 0.4        | 0.8 ± 0.5   | 0.7415         | 0.0444                          | 0.0128                               |
|   | Male   | 0.9 ± 0.4   | 0.9 ± 0.4   | 0.4857         | 0.9 ± 0.3        | 0.7 ± 0.4   | 0.0731         | 0.6693                          | 0.3012                               |
|   | Total  | 1.1 ± 0.4   | 1.1 ± 0.4   | 0.9776         | 0.9 ± 0.3        | 0.8 ± 0.3   | 0.3985         | 0.0794                          | 0.0081                               |
| Carbohydrate oxidation rate (mg/min/kg)                   | Female | 1.5 ± 0.5   | 0.9 ± 0.6   | 0.0139         | 2.2 ± 1.1        | 1.9 ± 0.9   | 0.4605         | 0.0332                          | 0.0167                               |
|   | Male   | 2.1 ± 1.2   | 1.3 ± 0.7   | 0.0812         | 1.7 ± 0.8        | 2.2 ± 0.8   | 0.1685         | 0.4158                          | 0.0288                               |
|   | Total  | 1.8 ± 0.9   | 1.1 ± 0.7   | 0.0063         | 1.9 ± 0.9        | 2.0 ± 0.9   | 0.7586         | 0.5743                          | 0.0008                               |

Data are expressed as mean ± SD.

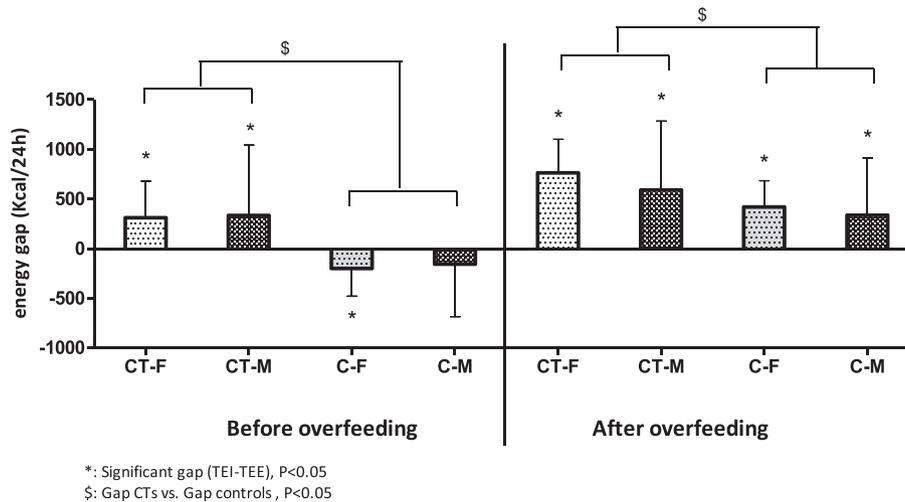
CT, constitutional thinness.

\**P* < 0.05 between TEI and TEE (gap) for each group in each condition at each time of the study.

**Table 3** Nitrogen balance assessed in two conditions: in free living and in calorimetric chamber

| Parameters           | Baseline                                  |              |               |               | Post-overfeeding |                |                    |                         |         |
|----------------------|---|--------------|---------------|---------------|------------------|----------------|--------------------|-------------------------|---------|
|                      | CTs                                       | Controls     | P value       | CTs           | Controls         | P value        | Time P value (CTs) | Time P value (controls) |         |
| Free living          | Mean carbohydrates intake (g)             | Female       | 238.0 ± 42.1  | 201.1 ± 32.5  | 0.0250           | 305.1 ± 44.6   | 279.3 ± 38.3       | 0.0001                  | <0.0001 |
|                      |   | Male         | 284.6 ± 80.7  | 269.0 ± 73.7  | 0.6595           | 308.0 ± 36.0   | 351.6 ± 78.9       | 0.1141                  | 0.0034  |
|                      |   | Total        | 260.3 ± 66.4  | 230.2 ± 62.7  | 0.1306           | 306.5 ± 39.8   | 312.2 ± 69.2       | 0.7350                  | 0.0012  |
|                      | Mean fat intake (g)                       | Female       | 84.4 ± 14.5   | 73.8 ± 18.7   | 0.1328           | 99.8 ± 9.7     | 96.9 ± 12.4        | 0.5377                  | 0.0068  |
|                      |   | Male         | 91.9 ± 21.8   | 84.3 ± 19.5   | 0.4116           | 95.8 ± 18.2    | 113.1 ± 21.1       | 0.0585                  | 0.5428  |
|                      |   | Total        | 88.0 ± 18.3   | 78.5 ± 19.3   | 0.0997           | 97.9 ± 14.2    | 104.3 ± 18.4       | 0.1972                  | 0.0197  |
|                      | Mean protein intake (g/day)               | Female       | 72.67 ± 13.24 | 71.33 ± 14.01 | 0.8129           | 101.50 ± 7.94  | 100.67 ± 6.50      | 0.7810                  | <0.0001 |
|                      |   | Male         | 83.55 ± 20.41 | 90.80 ± 22.30 | 0.4459           | 102.55 ± 18.23 | 124.50 ± 17.96     | 0.0120                  | 0.0060  |
|                      |   | Total        | 77.87 ± 17.55 | 80.18 ± 20.35 | 0.6848           | 102.00 ± 13.52 | 111.50 ± 17.55     | 0.0476                  | <0.0001 |
|                      | Mean protein intake per weight (g/kg/day) | Female       | 1.74 ± 0.32   | 1.16 ± 0.23   | <0.0001          | 2.35 ± 0.18    | 1.60 ± 0.16        | <0.0001                 | <0.0001 |
|                      |   | Male         | 1.56 ± 0.36   | 1.22 ± 0.32   | 0.0345           | 1.89 ± 0.37    | 1.64 ± 0.30        | 0.1116                  | 0.0106  |
|                      | N intake (g/day)                          | Total        | 1.65 ± 0.34   | 1.18 ± 0.27   | <0.0001          | 2.13 ± 0.37    | 1.62 ± 0.22        | <0.0001                 | <0.0001 |
| Female               |   | 11.63 ± 2.12 | 11.41 ± 2.24  | 0.8129        | 16.24 ± 1.27     | 16.11 ± 1.04   | 0.7810             | <0.0001                 |         |
| Male                 |   | 13.37 ± 3.27 | 12.73 ± 3.57  | 0.4459        | 16.41 ± 2.92     | 19.92 ± 2.87   | 0.0120             | 0.0060                  |         |
| N losses (g/day)     | Total                                     | 12.46 ± 2.81 | 12.83 ± 3.26  | 0.6848        | 16.32 ± 2.16     | 17.84 ± 2.81   | 0.0476             | <0.0001                 |         |
|                      | Female                                    | 7.26 ± 2.38  | 11.41 ± 3.64  | 0.0032        | 10.90 ± 3.69     | 12.73 ± 2.30   | 0.1598             | 0.0243                  |         |
|                      | Male                                      | 9.70 ± 3.85  | 14.14 ± 4.19  | 0.0202        | 11.66 ± 4.79     | 14.56 ± 3.10   | 0.1208             | 0.1645                  |         |
| N balance (g/day)    | Total                                     | 8.43 ± 3.33  | 12.65 ± 4.05  | 0.0004        | 11.27 ± 4.17     | 13.56 ± 2.79   | 0.0364             | 0.3369                  |         |
|                      | Female                                    | 4.37 ± 3.11  | 0 ± 2.84      | 0.0016        | 5.34 ± 3.95      | 3.38 ± 2.20    | 0.1470             | 0.0010                  |         |
|                      | Male                                      | 3.67 ± 4.07  | 0.39 ± 3.30   | 0.0582        | 4.75 ± 4.88      | 5.36 ± 3.85    | 0.7532             | 0.5167                  |         |
| Calorimetric chamber | Mean protein intake (g/day)               | Total        | 4.03 ± 3.53   | 0.18 ± 2.99   | 0.0003           | 5.06 ± 4.33    | 4.28 ± 3.15        | 0.4969                  | <0.0001 |
|                      |   | Female       | 67.37 ± 7.50  | 73.12 ± 7.82  | 0.0797           | 95.44 ± 8.97   | 104.68 ± 6.53      | 0.0086                  | <0.0001 |
|                      |   | Male         | 71.33 ± 8.99  | 76.79 ± 7.10  | 0.1420           | 97.12 ± 10.54  | 106.41 ± 7.39      | 0.0311                  | <0.0001 |
|                      | Mean protein intake per weight (g/kg/day) | Total        | 69.27 ± 8.31  | 74.79 ± 7.56  | 0.0246           | 96.24 ± 9.52   | 105.47 ± 6.82      | 0.0006                  | <0.0001 |
|                      |   | Female       | 1.61 ± 0.22   | 1.18 ± 0.11   | <0.0001          | 2.23 ± 0.21    | 1.66 ± 0.10        | <0.0001                 | <0.0001 |
|                      |   | Male         | 1.34 ± 0.20   | 1.02 ± 0.14   | 0.0007           | 1.78 ± 0.22    | 1.39 ± 0.16        | 0.0002                  | <0.0001 |
|                      | N intake (g N/day)                        | Total        | 1.48 ± 0.25   | 1.11 ± 0.14   | <0.0001          | 2.01 ± 0.31    | 1.54 ± 0.19        | <0.0001                 | <0.0001 |
|                      |   | Female       | 10.78 ± 1.20  | 11.67 ± 1.25  | 0.0797           | 15.27 ± 1.44   | 16.75 ± 1.05       | 0.0086                  | <0.0001 |
|                      |   | Male         | 11.41 ± 1.44  | 12.29 ± 1.14  | 0.1420           | 15.54 ± 1.67   | 17.03 ± 1.18       | 0.0311                  | <0.0001 |
|                      | N losses (g N/day)                        | Total        | 11.08 ± 1.33  | 11.97 ± 1.21  | 0.0246           | 15.40 ± 1.52   | 16.88 ± 1.09       | 0.0006                  | <0.0001 |
|                      |   | Female       | 8.74 ± 1.35   | 9.81 ± 1.79   | 0.1135           | 12.14 ± 3.24   | 13.26 ± 1.59       | 0.2924                  | 0.0044  |
|                      |   | Male         | 10.33 ± 1.60  | 12.98 ± 2.54  | 0.0093           | 13.25 ± 2.12   | 16.18 ± 2.65       | 0.0111                  | <0.0001 |
| N balance (g N/day)  | Total                                     | 9.50 ± 1.66  | 11.25 ± 2.66  | 0.0108        | 12.67 ± 2.76     | 14.59 ± 2.55   | 0.0200             | <0.0001                 |         |
|                      | Female                                    | 2.04 ± 0.91  | 1.89 ± 1.92   | 0.8124        | 3.13 ± 3.00      | 3.49 ± 1.84    | 0.7266             | 0.1287                  |         |
|                      | Male                                      | 1.08 ± 1.61  | -0.70 ± 2.46  | 0.0624        | 2.29 ± 1.90      | 0.85 ± 2.48    | 0.1486             | 0.0031                  |         |
| Total                | 1.58 ± 1.35                               | 0.71 ± 2.50  | 0.1531        | 2.73 ± 2.49   | 2.29 ± 2.49      | 0.5552         | 0.0052             |                         |         |

N intake was assessed with self-reported diet diaries (free living) and controlled weighed food by dietician (chamber), with 6.25 g of protein per grams of N. N losses was calculated based on Lee and Hartley formula: nitrogen excretion (g N/24 h) = urinary urea (mmol/24 h) × 0.028 × 1.2 (factor of non-urea urine N losses). A 30 g of protein provided by Renutryl gives 4.8 g of N on top of daily N intake during the overfeeding period. CT, constitutional thinness; N, nitrogen.



**Figure 1** Energy gap in free living in all groups before and after overfeeding. Data are expressed as mean  $\pm$  SD. \*significant gap (TEI-TEE),  $P < 0.05$  and  $^{\$}$ gap CTs vs. gap controls,  $P < 0.05$ .

nitrogen balance was found positive and significantly increased in CTs compared to their counterparts (Table 3).

Baseline free-living REE was significantly lower in CTs than in controls in both genders (Table 2). Once adjusted to LM, REE/LM was significantly greater in CTs men only as compared with their controls ( $31.7 \pm 3.1$  kcal/kg vs.  $28.0 \pm 3.3$  kcal/kg,  $P = 0.004$ ). TEE, assessed in both conditions (free living and chamber), was significantly lower in CTs compared to controls in both genders (Table 2).

Fasting fat oxidation was similar between CTs and controls, whereas fasting carbohydrate oxidation was higher in CTs women than in their controls (Table 2).

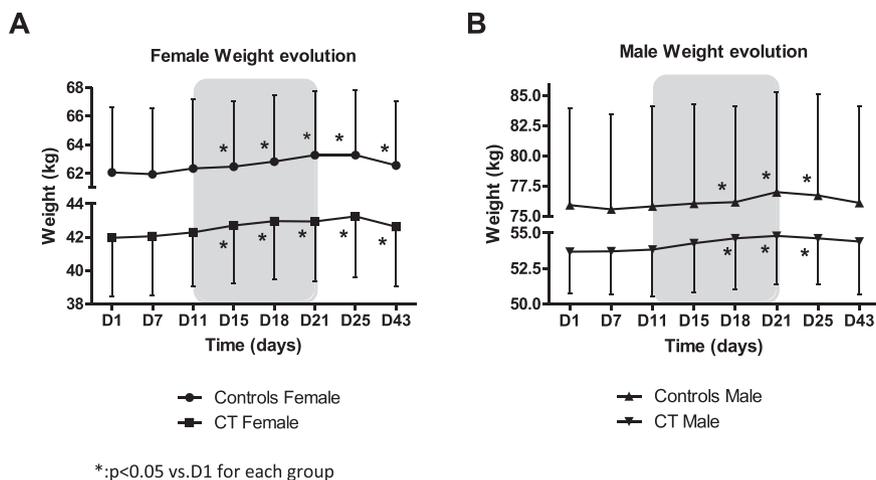
A positive energy gap was found in CTs in free-living conditions, in both genders. Controls tended to present a negative gap (Table 2 and Figure 1). In experimental condition (i.e. 24 h stay in the calorimetric chamber), we found a positive

gap only in female CTs and a negative one in male controls (Table 2).

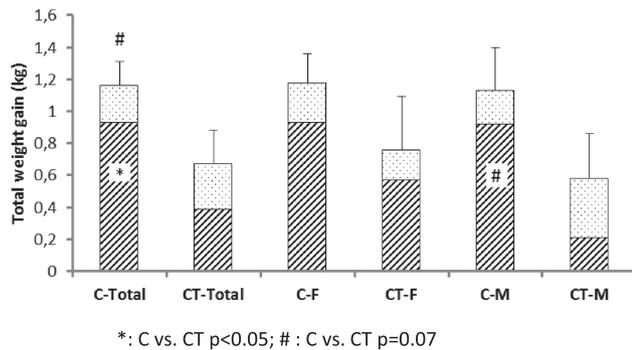
Correlation analysis between energy gap and baseline parameters in free-living showed strong correlation with snack calorie intake ( $P = 0.0002$ ,  $R^2 = 0.22$ ) and relative protein intake expressed per bodyweight (kg) ( $P < 0.0001$ ,  $R^2 = 0.512$ ).

### Energy and metabolic response to overfeeding (Table 2)

The short-term protein-energy overfeeding paradigm induced a bodyweight gain in all groups. The gained weight was maintained after 2 week post-overfeeding only in women (Figure 2). Overall calculated total bodyweight gain ( $TBW_{V8} - TBW_{V2}$ ) trend to be higher in controls than in CT ( $1.16 \pm 0.15$  vs.



**Figure 2** Weight changes in all groups throughout the study. Data are expressed as mean  $\pm$  SD. \* $P < 0.05$  vs. D1 in each group.



**Figure 3** Total body weight, lean mass, and fat mass gain comparison. Data are presented as mean  $\pm$  SD; hatched bars—lean mass; dotted bars—fat mass. Statistical analysis: \*C vs. CT  $P < 0.05$ ; #C vs. CT  $P = 0.07$ .

$0.68 \pm 0.21$ ,  $P = 0.07$ ) but not when considering each gender subgroup separately (Figure 3).

Body composition analyses revealed that LM significantly increased in controls in both genders but not in CTs. A trend to increase was noticed in CT women ( $P = 0.07$ ). Overall calculated LM gain ( $LM_{V8} - LM_{V2}$ ) was significantly higher in controls than in CT ( $0.93 \pm 0.16$  vs.  $0.39 \pm 0.19$  kg,  $P = 0.04$ ) and trend to be higher male controls ( $0.92 \pm 0.26$  vs.  $0.21 \pm 0.26$  kg,  $P = 0.07$ ). FM increased significantly in both groups but no differences in calculated FM gain ( $FM_{V8} - FM_{V2}$ ) were found between groups.

Total energy intake increased significantly during the overfeeding period in all study groups and study conditions. TEE remained stable in all groups in free-living conditions. Explored in the chamber stay, TEE significantly increased in controls only. As a result, energy gap was significantly increased by the overfeeding regimen in both CTs and controls (Table 2 and Figure 1).

In both study conditions, protein intake significantly increased in all groups, and the intake per bodyweight remained significantly higher in CTs compared to controls. Nitrogen losses were significantly increased in CTs subsequent to the intervention, yet the values remained lower to those of controls. The intervention affected strongly the nitrogen balance in controls, with significant positive changes. In these conditions the nitrogen balance became similar between CTs and controls (Table 3).

The overfeeding regimen shifted the preferential substrate oxidation at fasting state in CT women and in Controls by increasing carbohydrate oxidation rate (average of 1.35-fold increase) and decreasing fat oxidation (average of 0.84-fold decrease).

Fasting total and acylated ghrelin, PYY and GLP-1 levels were similar between CTs and controls before and after overfeeding period (Table 4 and Figure 4). The overfeeding intervention did not affect these hormonal parameters within each group. Test meal induced an acute postprandial fall of total and acylated ghrelin, similar in CTs and controls at baseline. The overfeeding intervention decreased

significantly the mean total ghrelin<sub>0-150min</sub> in CTs and controls. Mean PYY<sub>0-150min</sub> and mean GLP-1<sub>0-150min</sub> were significantly elevated in CTs as compared with controls before and after overfeeding. Likewise, we observed that the intervention tended to induce an early secretory response of PYY to the test meal in CTs.

### Urinary metabolomics identifies CT-specific metabolism (Figure 5)

Orthogonal partial least squares discriminant analysis models on total urine content normalization described metabolic differences between CT and controls at baseline. CT vs. controls differences in metabolome remained but were attenuated during the overfeeding period. Similar observations were achieved with data normalized to creatinine. Major/essential amino acids and central energy metabolism intermediates were present in higher concentrations in 24 h urine samples in CT compared with control subjects. The urinary metabolic phenotypic differences were not affected by the intervention.

## Discussion

In the present study, we describe how the CT condition is characterized by a non-malnourished state of underweight phenotype in females<sup>1,4,13</sup> but also in male subjects. Indeed, both genders of CT exhibited no eating disorders traits, normal values of nutritional biomarkers (IGF-1, free T<sub>3</sub>), and no excessive daily physical activity. Besides, both genders of CT displayed a lower percentage of body fat mass as compared with normal-weight controls, yet the values remained in the healthy range of body fat.<sup>31</sup> In line with our previous study,<sup>13</sup> CT's eating behaviour was associated in both genders to higher daily snacking episodes which accounted for non-negligible caloric contribution of snacking in TEI.

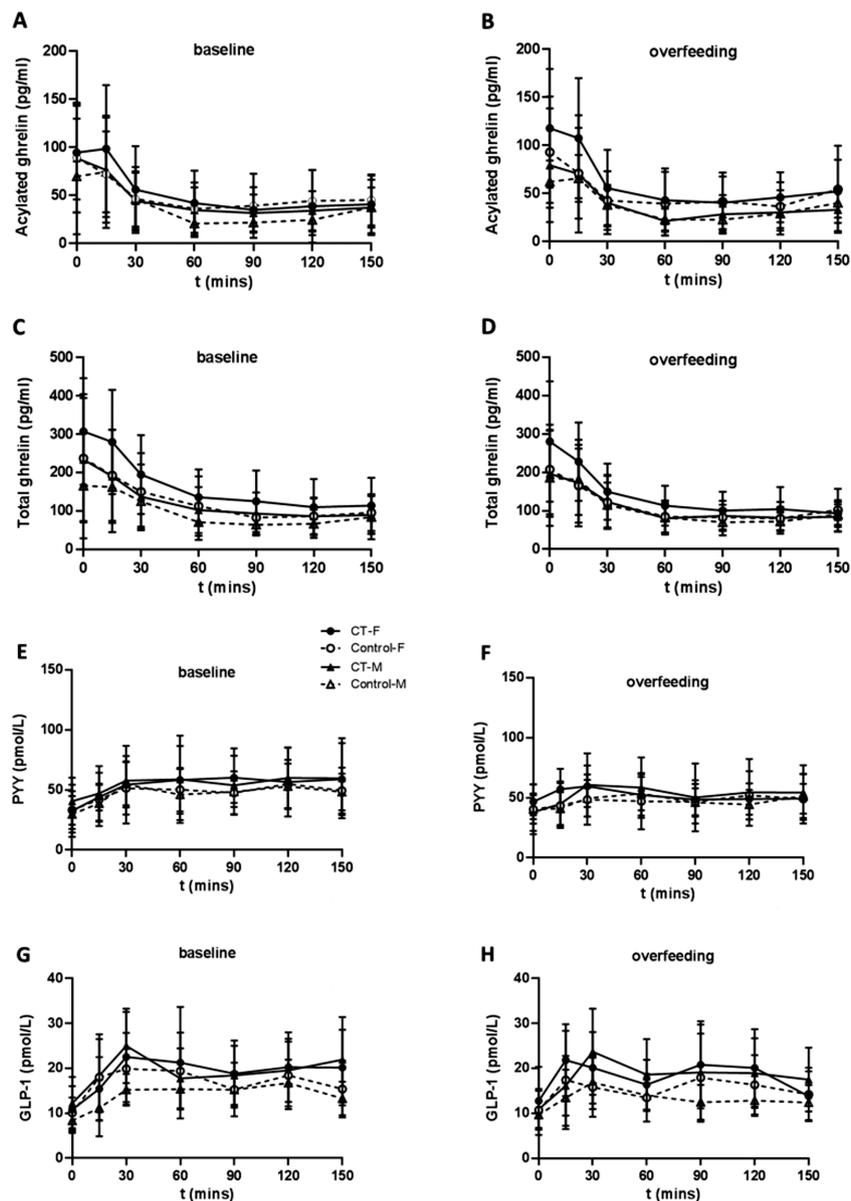
We report a paradoxical positive energy gap in CT male and female participants, confirming previous findings in women,<sup>13</sup> and discuss this phenotypic trait in relation to dietary, biochemical, and energy factors. No misreporting in food intake was found in our study, except for the common diet underreporting in normal-weight women in line with usual restrained eating scores as compared with a previous study.<sup>2</sup> The energy gap observed in CT individuals does not seem to be subject to a potential bias from our study design. This gap, shown in free-living conditions, was also confirmed in a controlled setting in CT women by using calorimetric chambers assessment. Taken all this, and in accordance with our usual energy equations, this paradoxical positive energy gap related to this particular CT phenotype might be necessary to prevent them from losing weight. Overfeeding ultimately led to weight gain in the CTs, although nominally

**Table 4** Fasting and postprandial profiles of appetite-regulatory hormones at baseline and at post-overfeeding

| Parameters                                | Baseline |               |         | Post-overfeeding |               |         |                    |                         |
|---|----------|---------------|---------|------------------|---------------|---------|--------------------|-------------------------|
|   | CTs      | Controls      | P value | CTs              | Controls      | P value | Time P value (CTs) | Time P value (controls) |
| Fasting total ghrelin (pg/mL)             | Female   | 267.2 ± 116.7 | 0.6406  | 274.5 ± 173.0    | 207.3 ± 116.9 | 0.2836  | 0.3882             | 0.4815                  |
|   | Male     | 234.0 ± 160.6 | 0.4449  | 196.5 ± 112.3    | 168.1 ± 124.2 | 0.5891  | 0.2051             | 0.4757                  |
|   | Total    | 249.8 ± 138.9 | 0.4093  | 235.5 ± 147.8    | 189.5 ± 119.0 | 0.2621  | 0.1218             | 0.3470                  |
| Fasting acylated ghrelin (pg/mL)          | Female   | 90.7 ± 40.1   | 0.9530  | 104.0 ± 62.9     | 93.1 ± 57.8   | 0.6687  | 0.9257             | 0.7225                  |
|   | Male     | 88.8 ± 56.4   | 0.4642  | 79.4 ± 59.3      | 62.3 ± 22.1   | 0.4024  | 0.3438             | 0.6721                  |
|   | Total    | 89.7 ± 48.1   | 0.5750  | 91.7 ± 60.9      | 79.1 ± 46.9   | 0.4470  | 0.4596             | 0.8818                  |
| Fasting PYY (pmol/mL)                     | Female   | 34.7 ± 22.5   | 0.8527  | 47.3 ± 15.2      | 40.3 ± 20.8   | 0.3755  | 0.0840             | 0.2075                  |
|   | Male     | 40.5 ± 19.7   | 0.1777  | 38.0 ± 15.6      | 40.3 ± 11.9   | 0.7098  | 0.6298             | 0.0837                  |
|   | Total    | 37.6 ± 20.9   | 0.2808  | 42.6 ± 15.8      | 40.3 ± 17.0   | 0.6418  | 0.3294             | 0.0303                  |
| Fasting GLP-1 (pmol/mL)                   | Female   | 10.2 ± 4.4    | 0.9266  | 12.1 ± 7.4       | 10.7 ± 4.3    | 0.5980  | 0.1063             | 0.5367                  |
|   | Male     | 12.2 ± 5.9    | 0.0686  | 10.9 ± 4.5       | 9.7 ± 2.9     | 0.4743  | 0.5276             | 0.0955                  |
|   | Total    | 11.2 ± 5.2    | 0.1394  | 11.5 ± 6.1       | 10.2 ± 3.7    | 0.4107  | 0.4411             | 0.1381                  |
| Test meal mean Total ghrelin (pg/L)       | Female   | 162.1 ± 112.7 | 0.2404  | 144.6 ± 110.8    | 121.2 ± 81.6  | 0.1309  | 0.0029             | 0.0472                  |
|   | Male     | 133.1 ± 103.2 | 0.4059  | 118.1 ± 82.5     | 105.4 ± 75.0  | 0.3355  | 0.0172             | 0.0691                  |
|   | Total    | 147.4 ± 108.6 | 0.1829  | 131.2 ± 98.1     | 114.0 ± 78.8  | 0.0934  | 0.0001             | 0.0075                  |
| Test meal mean acylated ghrelin (pmol/mL) | Female   | 56.1 ± 49.1   | 0.6941  | 54.8 ± 45.6      | 54.3 ± 43.3   | 0.9412  | 0.2715             | 0.8227                  |
|   | male     | 49.7 ± 41.8   | 0.2614  | 43.3 ± 41.8      | 40.1 ± 23.9   | 0.5705  | 0.0333             | 0.6177                  |
|   | Total    | 52.9 ± 44.8   | 0.3442  | 49.1 ± 44.0      | 47.8 ± 36.3   | 0.7823  | 0.0256             | 0.7933                  |
| Test meal mean PYY (pmol/mL)              | Female   | 52.9 ± 28.5   | 0.1180  | 51.2 ± 15.8      | 46.6 ± 20.0   | 0.1104  | 0.2449             | 0.8640                  |
|   | Male     | 53.9 ± 26.7   | 0.0299  | 45.6 ± 17.9      | 46.6 ± 14.3   | 0.1481  | 0.3006             | 0.6405                  |
|   | Total    | 53.4 ± 27.6   | 0.0089  | 46.3 ± 19.0      | 46.6 ± 17.6   | 0.0307  | 0.8833             | 0.9982                  |
| Test meal mean GLP-1 (pmol/L)             | Female   | 17.5 ± 9.0    | 0.4686  | 17.3 ± 7.7       | 15.1 ± 7.5    | 0.0656  | 0.6731             | 0.0043                  |
|   | Male     | 19.0 ± 8.1    | 0.0001  | 17.9 ± 8.4       | 13.1 ± 4.7    | <0.0001 | 0.0705             | 0.3426                  |
|   | Total    | 18.3 ± 8.6    | 0.0006  | 17.6 ± 8.0       | 14.1 ± 6.4    | <0.0001 | 0.1322             | 0.0047                  |

Data are expressed as mean ± SD.

CT, constitutional thinness; GLP-1, glucagon-like peptide-1; PYY, peptide tyrosine tyrosine.



**Figure 4** Post-test meal kinetic changes in appetite-regulatory hormones prior to overfeeding and at the end of the overfeeding in the constitutional thinness group (CT, dotted lines) and the control group (controls, plain lines). (A) Acylated ghrelin before and (B) after overfeeding; (C) Total ghrelin before and (D) after overfeeding; (E) PYY before and (F) after overfeeding; (G) GLP-1 before and (H) after overfeeding. Data are expressed as mean  $\pm$  SD. Statistical analysis: \* $P < 0.05$  vs.  $T_0$  min in each group. GLP-1, glucagon-like peptide 1; PYY, peptide tyrosine tyrosine.

lower than in the controls. This suggests that CTs' inability to gain weight might be overpassed at the cost of a particularly accentuated positive gap.

We further explored how this phenotype associates with distinct metabolic and energy states by considering the study of lipid, carbohydrate, and protein balance within basal and overfeeding conditions.<sup>32</sup>

Carbohydrate oxidation rate was higher in CT female subjects, which could be related to their higher food intake. The overfeeding intervention led to a decrease in fasting fat oxidation rate and increase in carbohydrate oxidation rate in all female subjects. This observation is in agreement with

previous studies reporting a decrease in fat oxidation in obesity-prone subjects with overfeeding<sup>33,34</sup> and a shift to carbohydrate instead of lipid oxidation upon lipid overfeeding of healthy men.<sup>34</sup>

CTs manifested a positive nitrogen balance that was markedly higher than in controls. First, it is important to note that CTs still consume similar amounts of food as controls and in particular proteins even though the protein needs should to be lower given their lower lean body mass. The positive nitrogen balance was mainly due to a lower nitrogen excretion compared with controls, suggesting a saturated lean mass protein turn over. We indeed recently reported that skeletal

| A | Metabolites                  | <sup>1</sup> H NMR chemical shift for variable | HMDB         | Chisq2 | p-value  | Adjusted p-value | B                          |         |         |         |         |                                   |  |  |
|---|------------------------------|--|--------------|--------|----------|------------------|----------------------------|---------|---------|---------|---------|-----------------------------------|--|--|
|   |                              |  |              |        |          |                  | Metabolites                |         |         |         |         | Fold of change between CT/control |  |  |
|   |                              |  |              |        |          |                  | Visit 1                    | Visit 3 | Visit 6 | Visit 7 | Visit 8 |                                   |  |  |
|   | Alpha-Hydroxyisobutyric acid | 1.36   | HMDB0000729  | 7.37   | 0.00662  | 0.01924          | 2-alpha-hydroxyisobutyrate | 1.05    | 1.06    | 1.01    | 1.06    | 1.09                              |  |  |
|   | Oxoglutaric acid             | 3.0  | HMDB0000208  | 19.89  | 8.20E-06 | 0.0001804        | 2-oxoglutaric acid         | 1.10    | 1.14    | 1.13    | 1.16    | 1.16                              |  |  |
|   | 3-Hydroxyisovaleric acid     | 1.27   | HMDB0000754  | 7.47   | 0.00628  | 0.0186           | 3-hydroxyisovaleric acid   | 1.16    | 1.16    | 1.13    | 1.26    | 1.03                              |  |  |
|   | Acetoacetic acid             | 2.30   | HMDB0000060  | 16.3   | 5.40E-05 | 0.000693         | Acetoacetate               | 1.15    | 1.13    | 1.17    | 1.23    | 1.17                              |  |  |
|   | L-Acetylcarnitine            | 2.14   | HMDB0000201  | 11.9   | 0.00056  | 0.002782         | Acetyl-carnitine           | 1.15    | 1.17    | 1.26    | 1.24    | 1.05                              |  |  |
|   | L-Alanine                    | 1.48   | HMDB0000161  | 7.79   | 0.00525  | 0.01617          | Alanine                    | 1.05    | 1.13    | 1.29    | 1.26    | 1.10                              |  |  |
|   | Allantoin                    | 5.41   | HMDB0000462  | 21.82  | 3.00E-06 | 9.24E-05         | Allantoin                  | 1.33    | 1.08    | 1.37    | 1.26    | 1.33                              |  |  |
|   | L-Arginine                   | 1.78   | HMDB0000517  | 7.18   | 0.00735  | 0.02058          | Arginine                   | 1.14    | 1.12    | 1.16    | 1.12    | 1.14                              |  |  |
|   | Creatine                     | 3.94   | HMDB0000064  | 7.58   | 0.00589  | 0.01779          | Creatine                   | 1.06    | 0.98    | 1.19    | 1.43    | 1.68                              |  |  |
|   | Dimethylamine                | 2.72   | HMDB0000087  | 13.47  | 0.00024  | 0.001422         | Dimethylamine              | 1.07    | 1.16    | 1.11    | 1.14    | 1.18                              |  |  |
|   | D-Glucose                    | 5.25   | HMDB0000122  | 16.46  | 5.00E-05 | 0.000693         | Glucose                    | 1.24    | 1.27    | 1.26    | 1.31    | 1.14                              |  |  |
|   | L-Glutamic acid              | 2.37   | HMDB0000148  | 23.31  | 1.40E-06 | 5.39E-05         | Glutamate                  | 1.08    | 1.17    | 1.16    | 1.22    | 1.13                              |  |  |
|   | L-Glutamine                  | 2.45   | HMDB0000641  | 16.38  | 5.20E-05 | 0.000693         | Glutamine                  | 1.10    | 1.13    | 1.20    | 1.21    | 1.10                              |  |  |
|   | Glycine                      | 3.57   | HMDB0000123  | 14.46  | 0.00014  | 0.001078         | Glycine                    | 1.37    | 1.44    | 1.48    | 1.43    | 1.22                              |  |  |
|   | L-Isoleucine                 | 0.95   | HMDB0000172  | 14.85  | 0.00012  | 0.001054         | Isoleucine                 | 1.11    | 1.15    | 1.10    | 1.13    | 1.20                              |  |  |
|   | L-Lactic acid                | 4.12   | HMDB0000190  | 13.35  | 0.00026  | 0.001483         | Lactate                    | 1.02    | 1.04    | 1.29    | 1.16    | 1.21                              |  |  |
|   | L-Leucine                    | 0.96   | HMDB0000687  | 17.62  | 2.70E-05 | 0.000462         | Leucine                    | 1.13    | 1.21    | 1.11    | 1.18    | 1.18                              |  |  |
|   | Methylmalonic acid           | 1.26   | HMDB0000202  | 10.98  | 0.00092  | 0.003978         | Methylmalonate             | 1.10    | 1.19    | 1.13    | 1.20    | 1.14                              |  |  |
|   | myo-Inositol                 | 3.52   | HMDB0000211  | 26.66  | 2.40E-07 | 1.23E-05         | Myo-inositol               | 1.23    | 1.20    | 1.21    | 1.20    | 1.13                              |  |  |
|   | N acetyl glycoprotein        | 2.07   | NA           | 14.58  | 0.00013  | 0.001054         | NAC                        | 1.18    | 1.10    | 1.16    | 1.15    | 1.06                              |  |  |
|   | O acetyl glycoproteins       | 2.08   | NA           | 8.01   | 0.00465  | 0.01492          | OAC                        | 1.17    | 1.13    | 1.19    | 1.13    | 1.06                              |  |  |
|   | Phosphocreatine              | 3.96   | HMDB00001511 | 15.38  | 8.80E-05 | 0.0009625        | Phospho creatine           | 1.05    | 1.15    | 1.11    | 1.24    | 1.12                              |  |  |
|   | Taurine                      | 3.44   | HMDB0000251  | 12.02  | 0.00053  | 0.002772         | Taurine                    | 1.20    | 1.30    | 1.14    | 1.20    | 1.11                              |  |  |
|   | L-Threonine                  | 4.24   | HMDB0000167  | 29.73  | 5.00E-08 | 6.39E-06         | Threonine                  | 1.22    | 1.20    | 1.13    | 1.16    | 1.19                              |  |  |
|   | L-Tryptophan                 | 7.28   | HMDB0000929  | 7      | 0.00813  | 0.02135          | Tryptophane                | 1.08    | 1.23    | 1.08    | 1.23    | 1.17                              |  |  |
|   | L-Tyrosine                   | 7.20   | HMDB0000158  | 6.13   | 0.01331  | 0.03254          | Tyrosine                   | 0.93    | 1.28    | 1.13    | 1.23    | 1.24                              |  |  |
|   | L-Valine                     | 1.00   | HMDB0000883  | 13.46  | 0.00024  | 0.001422         | Valine                     | 1.17    | 1.15    | 1.14    | 1.16    | 1.12                              |  |  |

| C                        | V2,V3 (baseline) | V6,V7,V8 (during overfeeding) |
|--------------------------|------------------|-------------------------------|
| Unnormalized             |                  |                               |
| R <sup>2</sup> X         | 0.16-0.17        | 0.14-0.18                     |
| R <sup>2</sup> Y         | 0.61-0.66        | 0.53-0.67                     |
| Q <sup>2</sup> Y         | 0.20-0.21        | <0-0.11                       |
| Normalized to creatinine |                  |                               |
| R <sup>2</sup> X         | 0.37-0.54        | 0.48-0.52                     |
| R <sup>2</sup> Y         | 0.46-0.52        | 0.39-0.44                     |
| Q <sup>2</sup> Y         | 0.11-0.25        | <0-0.13                       |

**Figure 5** Urinary metabolomics analyses: (A) urinary metabolite fold of change between CT and control subjects at the different visits; (B) results from analysis of variance on urine metabolite/creatinine ratios for a main effect of CT status; (C) parameters describing the orthogonal partial least squares discriminant analysis model, generated with one predictive and one orthogonal component, on total urine content. R<sup>2</sup>X is the explained variance in the urine metabolic profile, R<sup>2</sup>Y the explained group variance and Q<sup>2</sup>Y an indicator of mode robustness.

muscle energy metabolism was altered in CT women including a downregulation of cytoskeleton proteins and those involved in triglyceride storage or respiratory metabolism.<sup>15</sup> Oppositely to undernutrition or other catabolic situations associating a negative nitrogen balance,<sup>35,36</sup> a possible saturated protein turn over condition could lead in CTs to higher rates of amino acids flux. The findings generated through analysis of 24 h urine metabolomics provided evidence supporting this hypothesis. Indeed, we found higher urinary content in some essential amino acids including branched chain amino acids such as isoleucine, leucine, and valine but also threonine or tryptophan. Higher urinary levels of alanine and glutamine, both synthesized from branched chain amino acids in skeletal muscle and subsequently higher levels of arginine<sup>37</sup> suggest a global overload of protein turn over. Further physiological studies (especially with emphasis on total protein turn over) could provide deeper insights into the role of futile cycles in the CT phenotype. In particular, this metabolic trait seems to occur independently of overfeeding conditions and not to influence specific metabolic adaptation to overfeeding. Hence, skeletal muscle inefficiency might play a role for some energy loss that could be part of gap explanations. Altogether, we assumed that this particular skeletal muscle phenotype could be a susceptible factor of CTs

inability to gain lean mass contrary to controls after the overfeeding regimen. While this latter result should be interpreted with caution giving the small number of subjects some other data strengthen the concept of resistance to lean mass gain in CT. Indeed, CTs maintain a low lean mass in basal conditions, despite a high relative protein intake with more than 1.6 g/kg/day of protein intake, almost twice as the protein dietary intake recommendations in adults (0.8 g/kg/day).<sup>38</sup> This value is also higher than controls protein intake (1.2 g/kg/day) in our study, a value which fits the protein requirements in adults estimated by using an indicator amino acid oxidation technique.<sup>39</sup> CTs were still unable to increase their lean mass after overfeeding, whereas protein intake was raised to approximately 2.2 g/kg/day. These findings should be taken into account in clinical practice. While diagnosing sarcopenia in CT ageing patients or misdiagnosing CT as sarcopenia, a hypercaloric hyperprotein diet might be inefficient. We are currently evaluating whether an approach centred on physical activity could be useful to protect muscles in this particular population.

This high-protein consumption profile was associated with the postprandial anorectic tone found in CTs, in complete mirror image of obesity.<sup>40,41</sup> We confirmed, in both gender, a higher postprandial tone of PYY and GLP-1. We could

assume that this specific hormonal profile could be due to the high-protein consumption profile. This could be interesting to challenge CT patients by decreasing their protein intake in order to test if we could decrease PYY and GLP-1 postprandial rise. This hormonal and nutritional profile in CT could account for higher satiety feeling<sup>42</sup> and specific eating behaviour including smaller meals and frequent snacking<sup>43</sup> that should be interpreted as adaptive in this population.

Some limitations are raised by our study. Compliance to overfeeding protocol could be a major concern in this outpatient study. Currently, there is no reliable method to measure food intake in humans. Strict and frequent supervision by a dietician was therefore completed to ensure accurate food intake data and no over reporting was noted. It is very interesting to note that male participants appeared to be less prone than women to comply with the diet. Some indirect markers such as protein plasma level and nitrogen balance were used to check for accuracy of food intake reporting. Only participants who really complied with the overfeeding diet were included in statistical analyses using strict criteria. The subsequent small number of the participants may explain the lack of power in some results especially when analysis was performed for each gender.

To conclude, the blunted muscle energy mechanism, previously described in CTs in free-living state, is associated with basal saturated protein turn over suggested by the concordance of positive nitrogen balance and an increased urine excretion of essential amino acids. Most interestingly the fact that this saturation cannot be overpassed when enhancing this spontaneous high-protein intake supports the concept of the resistance to lean mass gain in this phenotype. Specific isotopic studies on amino acids turn over are needed to deeper explore this uncommon protein metabolism profile. CT patients profile should be taken into account all over their lives in order to avoid ineffective, aggressive therapies especially when clinical diagnosis of age related sarcopenia is evoked.

## Author contributions

Bogdan Galusca, Jorg Hager, Nele Gheldof, Bruno Estour, and Natacha Germain carried out the coordination and design of the study; Yiin Ling, Bogdan Galusca, Jacques Epelbaum, Dominique Grouselle, Yves Boirie, Christophe Montaurier, Joyceline Cuenco, James S. Minnion, Thierry Thomas, Sylvie Mure, Simona Bartova, Sofia Moco, Francois-Pierre Martin,

Nele Gheldof, and Natacha Germain performed the data collection; Yiin Ling, Bogdan Galusca, and Jerome Carayol performed the statistical analysis; Yiin Ling, Bogdan Galusca, Bruno Estour, Francois-Pierre Martin, Nele Gheldof, and Natacha Germain carried out the manuscript writing. All authors took part in the writing and final editing of the manuscript. All authors have been given a copy of the manuscript, all have approved the final version of the manuscript, and all are prepared to take public responsibility for the work and share responsibility and accountability for the results.

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## Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1 Supporting Information

Data S2 Supporting Information

## Conflict of interest

François-Pierre Martin, Jérôme Carayol, Simona Bartova, Sofia Moco, Jorg Hager, and Nele Gheldof are employees of Nestlé SA. Yiin Ling, Bogdan Galusca, Jacques Epelbaum, Dominique Grouselle, Yves Boirie, Christophe Montaurier, Joyceline Cuenco, James S. Minnion, Thierry Thomas, Sylvie Mure, Bruno Estour, and Natacha Germain declare that they have no conflict of interest.

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