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ORIGINAL ARTICLE

Specific appetite, energetic and metabolomics responses to fat overfeeding in resistant-to-bodyweight-gain constitutional thinness

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BACKGROUND: Contrasting with obesity, constitutional thinness (CT) is a rare condition of natural low bodyweight. CT exhibits preserved menstruation in females, no biological marker of undernutrition, no eating disorders but a bodyweight gain desire. Anorexigenic hormonal profile with high peptide tyrosine tyrosine (PYY) was shown in circadian profile. CT could be considered as the opposite of obesity, where some patients appear to resist diet-induced bodyweight loss.
OBJECTIVE: The objective of this study was to evaluate appetite regulatory hormones in CTs in an inverse paradigm of diet-induced weight loss.
METHODS: A 4-week fat overfeeding (2640 kJ excess) was performed to compare eight CT women (body mass index (BMI) $<17.5\ \mathrm{kg\,m^{-2}}$) to eight female controls (BMI 18.5–25 $\mathrm{kg\,m^{-2}}$). Appetite regulatory hormones profile after test meal, food intake, bodyweight, body composition, energy expenditure and urine metabolomics profiles were monitored before and after overfeeding.
RESULTS: After overfeeding, fasting total and acylated ghrelin were significantly lower in CTs than in controls ($P=0.01$ and 0.03, respectively). After overfeeding, peptide tyrosine tyrosine (PYY) and glucagon-like-peptide 1 both presented earlier (T15 min vs T30 min) and higher post-meal responses (incremental area under the curve) in CTs compared with controls. CTs failed to increase bodyweight ($+0.22\pm0.18$ kg, $P=0.26$ vs baseline), contrasting with controls ($+0.72\pm0.26$ kg, $P=0.03$ vs baseline, $P=0.01$ vs CTs). Resting energy expenditure increased in CTs only ($P=0.031$ vs baseline). After overfeeding, a significant negative difference between total energy expenditure and food intake was noticed in CTs only (-2754 ± 720 kJ, $P=0.01$).
CONCLUSION: CTs showed specific adaptation to fat overfeeding: overall increase in anorexigenic hormonal profile, enhanced post prandial GLP-1 and PYY and inverse to controls changes in urine metabolomics. Overfeeding revealed a paradoxical positive energy balance contemporary to a lack of bodyweight gain, suggesting yet unknown specific energy expenditure pathways in CTs.

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INTRODUCTION

The obesity epidemic is partly explained by the obesogenic environment with abundant food supply and low physical activity. However, a high proportion of obese patients appears resistant to both calorie-controlled diets and drugs intervention strategies without any pathophysiological explanation to date.[1] Opposite to the high body mass index (BMI) of obesity, constitutional thinness (CT) is a natural state of underweight.[2–4] CT women often consult for bodyweight gain desire. Despite similar BMI to anorexia nervosa patients (AN), CTs do not exhibit any psychological or biological features adaptive to undernutrition of AN.[5] They display normal menstruation accounting for a normal nutritional status.[3] In basal conditions they have a balanced energy homeostasis including food intake and total energy expenditure comparable to that of normal BMI controls. Their bodyweight remains in the lower percentiles for gender and ethnicity throughout lifetime.[6]

Recent studies in CT patients indicate that many features, such as gut hormones regulating energy homeostasis,[7] have an opposite profile to that seen in obese patients, suggesting CT as an opposite condition to obesity. Orexigenic ghrelin is low in obese patients and increases after diet-induced bodyweight loss.[8] Anorexigenic peptides such as PYY and glucagon-like peptide 1 (GLP-1) are reduced/blunted in obese patients and increase after gastric by-pass surgery.[9–11] In CT subjects, ghrelin is in the normal range[12] and PYY is high in circadian profile.[3] This anorexigenic hormone profile could be considered as a constitutive and physiological factor integrating the underweight steady state of CT[12] despite living in the current obesogenic environment.

This study was designed to evaluate appetite regulatory hormones in CTs, by challenging them with a fat overfeeding paradigm, an inverse paradigm of diet-induced weight loss.[13] Appetite regulatory hormones after a test meal together with bodyweight, body composition, energy balance and metabolomics adaptations were therefore assessed in a CT group of women as well as in a control group, before and after a 4-week fat overfeeding period.

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SUBJECTS AND METHODS

Subjects

Sixteen female subjects were recruited in this outpatient setting study: eight CTs among outpatients consulting for bodyweight gain desire and eight controls recruited by advertising (BMI between 18.5 and 25 $kg\,m^{-2}$) matched by age (18–36 years). CTs exhibited the following criteria before inclusion: BMI between 13 and 17.5 $kg\,m^{-2}$, stable bodyweight throughout post-pubertal period; no amenorrhea; no AN or other eating disorders features confirmed by psychiatric evaluation and validated psychological scales (Eating Disorders Examination (EDE), Dutch Eating Behavior Questionnaire (DEBQ));[6] no under nutritional markers including normal IGF-1, estradiol, free triiodothyronine (FT3), mean cortisol and non-blunted leptin;[3] no hepatic disorders and no over exercise behavior according to the MONICA optional study of physical activity (MOSPA) questionnaire.[14] None of the subjects had documented chronic or congenital disease, none were taking any medication and none were smokers.

The clinical investigation was conducted in accordance with the Helsinki Declaration of 1975 (as revised in 1983). The local research and ethics committee of Saint-Etienne, France, approved the study and all subjects gave written informed consent before inclusion in the study. This study was registered at clinicaltrial.gov as NCT01224561.

Study design and dietary intervention

Participants were first screened for their usual diet and physical activity level during the run-in period from days 1 (inclusion) to 5. Then, both groups underwent a 4-week fat overfeeding intervention in outpatient setting (from days 5 to 34) consisting in 2640 kJ (630 kcal) excess daily, enough to induce a significant bodyweight gain in controls.[15] Subjects were provided with packages containing a fixed daily quantity of olive oil, peanuts, gruyere cheese and butter (31 ± 7.4% of saturated fatty acids, 52 ± 5.4% of mono unsaturated fatty acids and 17 ± 2.7% of poly unsaturated) to add to their usual daily diet. They were asked to maintain their normal lifestyle (baseline diet and physical activity) during the whole study. The dietary protocol was explained by a dietician at days 1 and 5. Scheduled appointments allowed to progressively deliver food packages, evaluate bodyweight and regularly check the compliance, in order to avoid compensatory behaviors.[16] Subjects were then allowed *ad libitum* food intake from days 34 to 62.

Each subject underwent a similar comprehensive examination at the three main visits of the study (days 5, 34 and 62): anthropomorphic analysis (bodyweight, height and body composition), energy balance evaluation, urine metabolomics analysis and appetite regulatory hormones profile assessment after test meal (for more details, see Supplementary Figure S1).

Body composition assessment

Body composition was measured at each main visit using three methods: quantification of the percentage of total body fat mass (FM) and fat-free mass (FFM) by dual-energy X-ray absorptiometry (DXA),[17] whole-body bioelectrical impedance (BODYSTAT Ltd; Isle of Man, UK)[18] and abdominal fat area by magnetic resonance imaging (Magnetom Symphonie 1.5 Tesla; Siemens AG, Munich, Germany).[19]

Energy balance assessment

Food intake was evaluated using a dietary daily-self-reporting record.[6] during 5 days, three times in the study (days 1–5, 17–21 and 29–34). Snacking was evaluated and defined as food eaten between the three official meals (breakfast, lunch and dinner). Over- and under-reporting were tracked using Goldberg equation[20,21] and defined as follows: underreporting = energy intake (EI)/resting energy expenditure (REE) < 1.4; overreporting = EI/REE > 2.4.

Resting energy expenditure (REE) was measured after a 12-hour overnight fast at each main visit by indirect calorimetry (Deltatrac TM, Datex Corp., Helsinki, Finland).[22] The activity energy expenditure (AEE) was measured in outpatient setting during 5 days before each main visit with an accelerometer (ACTIHEART, CamNtech, Cambridge, UK).[23] Total daily energy expenditure (TEE) was calculated as follows: $TEE = REE \times PAL$ (physical activity level measured by accelerometry).[24] Energy balance was estimated by calculating the energy gap as follows: Gap = TEE − daily total food intake.

Basal and test meal sampling

Venous samples were collected at 0700 hours after a 12-hour overnight fast to measure insulin, blood glucose, triglycerides, β-hydroxybutyrate, glycerol, free fatty acid (FFA), amino acid transferase (AAT), leptin, insulin-like growth factor 1 (IGF-1) and 17 beta estradiol. Twenty-four-hour mean cortisol was also evaluated as previously described.[3] Standardized test meals were performed at each main visit after a 12-hour overnight fast (1373 kJ, 328 kcal; 80% carbohydrates, 13% lipids and 7% proteins)[25] and blood samples were collected at T0, 15, 30, 60 and 90 min to measure insulin, blood glucose, total and acylated ghrelin, obestatin, PYY_{3-36} and total GLP-1. The test meal was fully eaten between T0 min and T15 min in the presence of a study investigator. Venous blood samples were then aliquoted and collected in glass tubes containing EDTA and aprotinin, immediately cold-centrifuged and kept frozen at −80 °C. For each hormone, all samples were assayed simultaneously.

Assays

Plasma blood glucose, triglycerides, FFA, AAT, insulin, cortisol, IGF-1, leptin and 17 beta estradiol were assayed using standardized techniques.[3] Obestatin, total and acylated ghrelin were determined by RIA kit (RK-031–30; Phoenix Pharmaceuticals, Belmont, CA, USA) at UMR 894 INSERM, Paris, France. Coefficients of variation for obestatin were 8 and 5% for ghrelin.[26] PYY_{3-36} was also measured in Paris, by enzyme immunoassays kit (Phoenix, Burlingame, CA, USA) with coefficients of variation 10%[26] and total GLP-1 by RIA (coefficient of variation $< 10\%$) as previously described at Imperial College, London, UK.[3]

Metabolomics analysis

Urinary metabolomics analysis was done at UMR 1019, Clermont-Ferrand, France.[27] Twenty-four-hour urine samples were collected at each main visit and homogenized. Samples (15 ml) were kept frozen at −20 °C in order to be analyzed simultaneously using a reversed phase UPLC chromatography system coupled to a time-of-flight mass spectrometer (TOF MS).[28] Metabolites with differential abundance between both groups and/or main visits were identified by *m/z* using METLIN (metlin.scripps.edu/), Human Metabolome Project (metabolomics.ca/) and KEGG ligand (genome.jp/kegg.html) databases)[27,29] (for more details, see Supplementary Method S2).

Statistical analysis

Sixteen subjects were sufficient to reveal a significant bodyweight gain with a 90% power calculation and 0.05 as an α-risk.[15,30,31] Data are presented as mean ± s.e.m. Incremental area under the curve (iAUC) for gut peptides at each test meal was obtained by subtracting the rectangle corresponding to basal value multiplied by 90 min from total AUC calculated using the trapezoidal rule.

Mann–Whitney's non-parametric tests were used to compare one-time measured characteristics including each point of test meal between the groups at each visit. Wilcoxon signed rank's non-parametric tests were used to analyze the differences between food intake and total energy expenditure in each group at days 5 and 34.

A one-factor (time) repeated measures ANOVA was used to analyze the parameters changes over the visits, including mean values of appetite regulatory hormones over each test meal and appetite regulatory hormones changes during each test meal (expressed as percentage change from baseline at each visit) in each group. Fischer's PLSD *post hoc* test was performed when time effect was significant.

A two-factor (group and time) repeated measures ANOVA was also performed for metabolomics on all extracted ions using PROC-MIXED procedure of SAS software v9.1 (SAS Institute Inc., Cary, NC, USA). Partial least square analysis (PLS) with orthogonal signal correction (OSC-PLS) was performed using SIMCAP+ v12 software (Umetrics, Umeå, Sweden). Significant ions for interaction were also analyzed by hierarchical clustering analysis.[32]

The *P*-value significance threshold was set to 0.05. All these statistical analyses and graphs were performed with StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA, USA) and GraphPad Prism 5.0 Softwares (GraphPad Software, San Diego, CA, USA).

RESULTS

Baseline characteristics

CTs were comparable to controls in both age (21.6 ± 1.9 vs 22.1 ± 0.8 years, $P = 0.6$) and height (1.61 ± 0.03 vs 1.64 ± 0.02 m, $P = 0.5$). CTs displayed a significantly lower bodyweight, BMI, total

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FM (Table 1) and plasma leptin than controls (8.3 ± 1.4 vs $12.6 \pm 0.9\,\mu g\,l^{-1}$, $P = 0.04$), but were similar to controls for IGF1 (293 ± 32 vs $285 \pm 18\,UI\,l^{-1}$, $P = 0.8$), cortisol (220 ± 18 vs $252 \pm 18\,nmol\,l^{-1}$, $P = 0.5$), 17 beta estradiol (70.1 ± 8.9 vs $53.6 \pm 11.6\,ng\,l^{-1}$, $P = 0.6$), free T3 (3.8 ± 0.1 vs $3.6 \pm 0.1\,pmol\,l^{-1}$, $P = 0.8$), fat and liver assessments (AAT: 24.5 ± 1.3 vs $28.5 \pm 2.8\,UI\,l^{-1}$, $P = 0.4$). Psychological scores showed no eating-disorder-related traits such as perfectionism, drive to thinness or excessive bodyweight/shape concern in CTs compared with controls. Moreover CTs exhibited lower restrained eating scores than controls (EDE score: 1.2 ± 0.5 vs 4.6 ± 1.2, $P = 0.03$; DEBQ score: 12.9 ± 1.8 vs 26.3 ± 2.7, $P = 0.010$; Table 1).

Dietary intervention monitoring

Daily energy intake and macronutrient distribution did not differ between the groups at any visits. Daily energy intake significantly increased after overfeeding compared to baseline in both groups. Analysis of daily meals distribution showed a significantly higher percentage of snacking in CTs compared with controls at baseline ($P = 0.045$) and on day 34 ($P = 0.002$) (for more details, see Supplementary Table S3). Monitored as markers of dietary intervention, triglycerides and FFA tended to increase on day 34 in both groups, and returned to baseline values on day 62. According to Goldberg equation, no over-reporting (EI/REE > 2.4) was noted in CTs (EI/REE = 2.1 ± 0.1) nor in controls (EI/REE = 1.7 ± 0.1) at the end of the overfeeding period (Table 1).

Effect of fat overfeeding on bodyweight

At the end of the overfeeding period (day 34), mean bodyweight of CTs was not different from baseline ($+0.225 \pm 0.180$ kg, $P = 0.26$ vs baseline), whereas controls' mean bodyweight was significantly increased compared with baseline value ($+0.725 \pm 0.268$ kg, $P = 0.03$ vs baseline). On day 62, CTs' mean bodyweight was decreased (-0.600 ± 0.224 kg, $P = 0.02$ vs day 34), while mean bodyweight gain remained significantly elevated in controls ($+0.800 \pm 0.281$ kg, $P = 0.02$ vs baseline and $P = 0.75$ vs day 34). Individual data for BMI are presented in Supplementary Figure S4 (Table 1).

Effect of fat overfeeding on appetite regulatory hormones

Total GLP-1. iAUC for GLP-1 remained stable in CTs and tended to decrease in controls after overfeeding (Figure 1). At day 34, iAUC for GLP-1 was higher in CTs than in controls ($P = 0.05$; Figure 1a).

Kinetic change analysis: at baseline, GLP-1 surged at T30 min ($P < 0.05$ vs T0 min) in both groups (Figure 1c). After overfeeding, the surge in total GLP-1 occurred earlier at T15 min ($P = 0.03$ vs T0) in both groups and GLP-1 increment persisted in CTs over the rest of the test meal (Figure 1d). This surge remained earlier at T15 min in both groups at day 62 (Figure 1e).

PYY3–36. IAUC for PYY_{3-36} also remained stable in CTs and decreased in controls after overfeeding ($P = 0.05$). At day 34, iAUC for PYY_{3-36} was higher in CTs than in controls ($P = 0.02$; Figure 1b).

Kinetic change analysis: at baseline, PYY_{3-36} surged at T30 min in CTs ($P < 0.05$ vs T0 min) and at T60 min in controls ($P < 0.05$ vs T0 min; Figure 1f). On days 34 and 62, CTs exhibited an earlier surge compared with baseline, at T15 min ($P < 0.05$ vs T0 min; Figures 1g and h). No surge was observed in controls at day 34.

Ghrelin/obestatin. Fasting plasma levels of total ($785 \pm 43\,pg\,ml^{-1}$ at day 34 vs $770 \pm 62\,pg\,ml^{-1}$ at day 5, $P = 0.6$) and acylated ghrelin ($195 \pm 25\,pg\,ml^{-1}$ at day 34 vs $255 \pm 32\,pg\,ml^{-1}$ at day 5,

Table 1. General parameter changes[a]

Parameters	CTs (n = 8)			Controls (n = 8)		
	Day 5[b]	Day 34	Day 62	Day 5	Day 34	Day 62
Body composition						
Bodyweight (kg)	44.6 ± 2.30[c,d]	44.8 ± 2.28[c]	44.2 ± 2.14[c]	59.2 ± 2.06	59.8 ± 2.16[e]	60.0 ± 2.19[e]
BMI (kg m^{-2})	17.1 ± 0.3[c]	17.1 ± 0.3[c]	16.9 ± 0.2[c]	22.1 ± 0.3	22.3 ± 0.3	22.3 ± 0.4
Fat mass by DXA (%)	24.2 ± 1.6[c]	25.0 ± 1.5[c]	23.7 ± 1.8[c]	30.2 ± 1.6	30.9 ± 1.5	31.1 ± 1.7[e]
Fat mass by impedance (%)	23.5 ± 2.3[c]	25.3 ± 2.0[c]	24.2 ± 1.6[c]	25.5 ± 1.2	27.7 ± 0.8[e]	28.1 ± 0.7[e]
Total abdominal fat by IRM (cm^3)	29.7 ± 5.5[c]	34.4 ± 6.5[c]	33.5 ± 6.0[c]	50.2 ± 10.3	52.2 ± 9.9	53.4 ± 11.3
Food intake						
Total daily energy intake (kJ day^{-1})	8198 ± 670	10 132 ± 716[e]	—	7 658 ± 444	10 069 ± 825[e]	—
Fat (g)	84 ± 7.7	132 ± 8.8[e]	—	69 ± 4.7	127 ± 7.6[e]	—
Snacking (% of total daily energy intake)	12.3 ± 1.9[c]	15.7 ± 2.1[c]	—	7.3 ± 1.5	7.7 ± 1.5	—
Energy expenditure						
Resting energy expenditure (REE; kJ day^{-1})	4748 ± 209[c]	4952 ± 205[c,e]	4714 ± 167[c]	5669 ± 134	5878 ± 184	5711 ± 159
REE/fat free mass (kJ day^{-1} kg^{-1})	144 ± 6	152 ± 5[c,e]	144 ± 4	142 ± 3	149 ± 3	144 ± 3
Respiratory quotient	0.88 ± 0.03	0.85 ± 0.01	0.84 ± 0.01	0.86 ± 0.02	0.87 ± 0.02	0.86 ± 0.01
Carbohydrate oxidation index (g day^{-1})	130 ± 24	114 ± 16	93 ± 17	136 ± 25	160 ± 25	148 ± 13
Fat oxidation index (g day^{-1})	25.2 ± 9.1	37.1 ± 7.4	40.7 ± 6.7	46.2 ± 9.5	40.7 ± 12.1	41.7 ± 6.7
Activity energy expenditure (kJ day^{-1})	6782 ± 720	8185 ± 1474	6117 ± 1088	6644 ± 945	8503 ± 1964	6975 ± 1193
Total energy expenditure (kJ day^{-1})	7348 ± 289	7775 ± 498	7222 ± 264	8135 ± 373	8654 ± 641	8206 ± 356
Gap (total EE—daily energy intake) (kJ)	− 1180 ± 523	− 2754 ± 720	—	+ 477 ± 447	− 1419 ± 887	—
Fat metabolism						
Triglycerides (mmol l^{-1})	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	0.6 ± 0.1
β OH butyrate (mmol l^{-1})	167 ± 37	207 ± 24	223 ± 33	121 ± 28	192 ± 90	133 ± 27
Glycerol (mmol l^{-1})	77.0 ± 9.3	59.3 ± 6.4	62.0 ± 7.7	54.1 ± 9.2	71.7 ± 14.9	60.7 ± 9.1
Free fatty acids (mmol l^{-1})	422 ± 41	551 ± 54	403 ± 25	303 ± 60	440 ± 102	365 ± 90

[a]Data are expressed as mean ± s.e.m. for CT ($n = 8$) and controls (C, $n = 8$). [b]Day 5 is baseline before overfeeding, day 34 is at the end of the 2640 kJ (630 kcal) per day excess overfeeding period and day 62 is after *ad libitum* food intake period. [c]$P < 0.05$ vs controls at each visit. One-factor (time) repeated measures ANOVA followed by *post hoc* analysis. [d]Statistics: Mann–Whitney's non parametric tests [e]$P < 0.05$ vs day 5 for each group.

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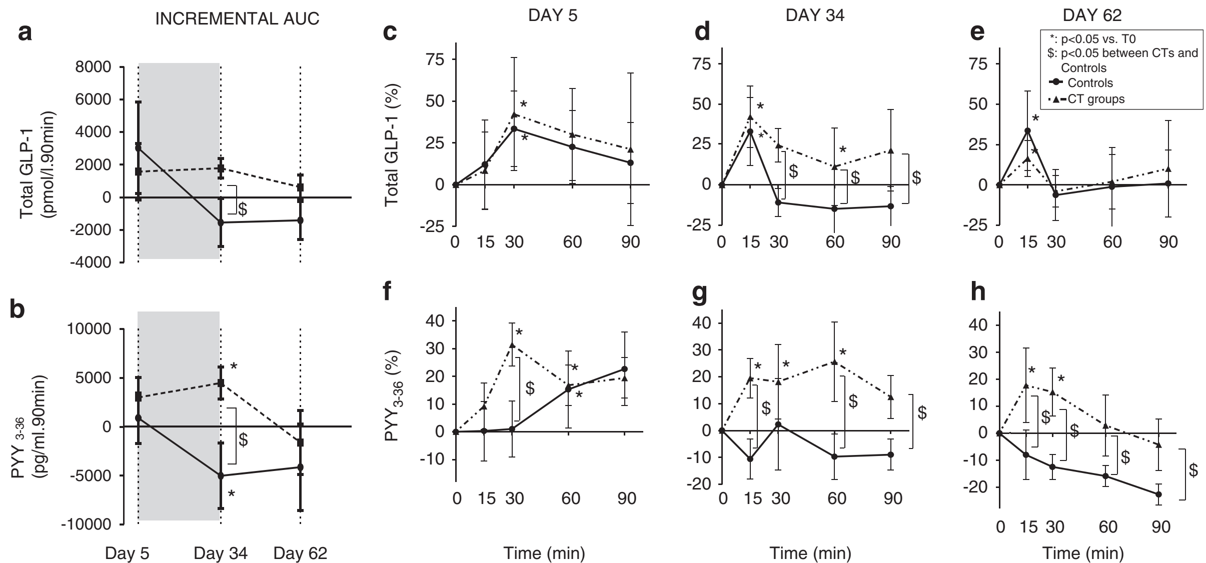

Figure 1. GLP-1 and PYY post-test meal kinetic changes. Post-test meal kinetic changes in anorexigenic gut hormones before overfeeding (day 5), at the end of the 2640 kJ (630 kcal) per day excess overfeeding period (day 34) and after *ad libitum* food intake period (day 62) in the CT group ($n = 8$, dotted line) and the control group (controls, $n = 8$, plain line). Standardized test meal (1373 kJ (328 kcal); 80% carbohydrates, 13% lipids and 7% proteins) was served and eaten between T0 min and 15 min. (**a**) iAUC for total GLP-1 expressed as pmol per l.90 min at each test meal (days 5, 34 and 62). (**b**) iAUC for PYY_{3-36} expressed as pg ml^{-1} per 90 min at each test meal. (**c**) Kinetic changes of total GLP-1 after test meal expressed as percentage change from baseline (T0) on days 5, (**d**) 34 and (**e**) 62. (**f**) Kinetic changes of PYY_{3-36} after test meal on days 5, (**g**) 34 and (**h**) 62. Data are expressed as mean ± s.e.m. Statistical analysis: *$P < 0.05$ vs T0 min in each group; $\$P < 0.05$ between CTs and controls.

$P = 0.4$) were not modified by overfeeding in CTs. Fasting total (914 ± 73 pg ml^{-1} at day 34 vs 711 ± 20 pg ml^{-1} at day 5, $P = 0.01$) and acylated ghrelin (321 ± 39 pg ml^{-1} at day 34 vs 204 ± 21 pg ml^{-1} at day 5, $P = 0.03$) increased after overfeeding in controls. Fasting total and acylated ghrelin were lower in CTs than in controls on day 34 ($P = 0.01$ and 0.03, respectively).

IAUC for total and acylated ghrelin increased in CTs ($P = 0.03$) and decreased in controls (respectively $P = 0.03$ and 0.02) after overfeeding. At day 34, iAUC for total and acylated ghrelin were higher in CTs than in controls (respectively $P = 0.02$ and 0.02; Figures 2a and b).

Kinetic change analysis: at baseline, total and acylated ghrelin tended to be suppressed by the meal in both groups (Figures 2d and g). After overfeeding, total and acylated ghrelin were significantly suppressed by the meal at T30 min ($P = 0.02$ and 0.03 vs T0 min, respectively) in controls but were not modified in CTs (Figures 2e and h). On day 62, total and acylated ghrelin were significantly suppressed by the meal in both groups at 30 min ($P = 0.02$; Figures 2f and i). Obestatin was significantly suppressed by the meal at each visit in both groups at the three visits (Figures 2j–l).

Effect of fat overfeeding on energy balance

REE and REE/FFM significantly increased in CTs on day 34 compared with baseline ($P = 0.03$ and $P = 0.04$ respectively), and returned to baseline on day 62 ($P = 0.67$ and $P = 0.16$ respectively). No significant change in REE and REE/FFM was detected in controls on days 34 or 62. AEE was comparable between groups at each visit and was not modified by the overfeeding (Table 1). At baseline, no difference was noticed between TEE and daily energy intake in both groups, showing a balanced energy homeostasis. At the end of the overfeeding, the difference between TEE and food intake (energy gap) was significant in CTs ($P = 0.01$), whereas it remained non-significant in controls. Energy gap significantly increased in CTs during the overfeeding ($P = 0.04$ vs baseline), whereas it remained stable in controls ($P = 0.35$ vs baseline; Table 1 and Figure 3).

Effect of fat overfeeding on urine metabolomics analysis

A total of 3 243 ions were extracted from urine analysis, including 94 ions significant for group effect, 84 ions for time effect and 542 ions for interaction effect (Figure 4). After noise reduction, OSC-PLS discriminated both group (axe 2) and interaction effect (axe 1), with a good fit of the models ($R^2Y = 0.65$, $Q^2cum = 0.62$). Results show a good discrimination of urine metabolomics at day 34, suggesting an important metabolic modification at this time for both groups with larger amplitude for CTs (Figure 4a).

When analyzed by hierarchical clustering analysis, two clusters with opposite profiles were found (heatmap, Figure 4b): one cluster included CTs on days 5 and 62 and controls on day 34 with the highest values (blue color) for the majority of the ions. The other cluster included controls on days 5 and 62 and CTs on day 34 with the lowest values (yellow color). For each group, metabolomics on day 62 was not significantly different from baseline showing a return to the initial state. During the overfeeding period measured on day 34, each metabolomics phenotype switched to the other group. We observed a significant decrease in identified metabolites in CTs, whereas an increase in controls, including metabolites from mitochondria such as phenylalanine ($P = 0.01$), tyrosine ($P = 0.005$) and xanthurenic acid ($P = 0.001$), and metabolites involved in FFA metabolism such as carnitine ($P = 0.03$; for more details, see Supplementary Figure S5).

DISCUSSION

CT is a rare condition of natural low bodyweight, with no sign of undernutrition and no eating disorders but a bodyweight gain desire. However, they seem to fail gaining bodyweight. Previous static evaluation of appetite regulatory hormones in CTs showed an overall anorexigenic tone that may contribute to their low stable bodyweight.[12] We therefore designed this study in a dynamic perspective by challenging CTs with a fat overfeeding. CTs displayed an enhanced anorexigenic response associated to a lack of bodyweight gain.

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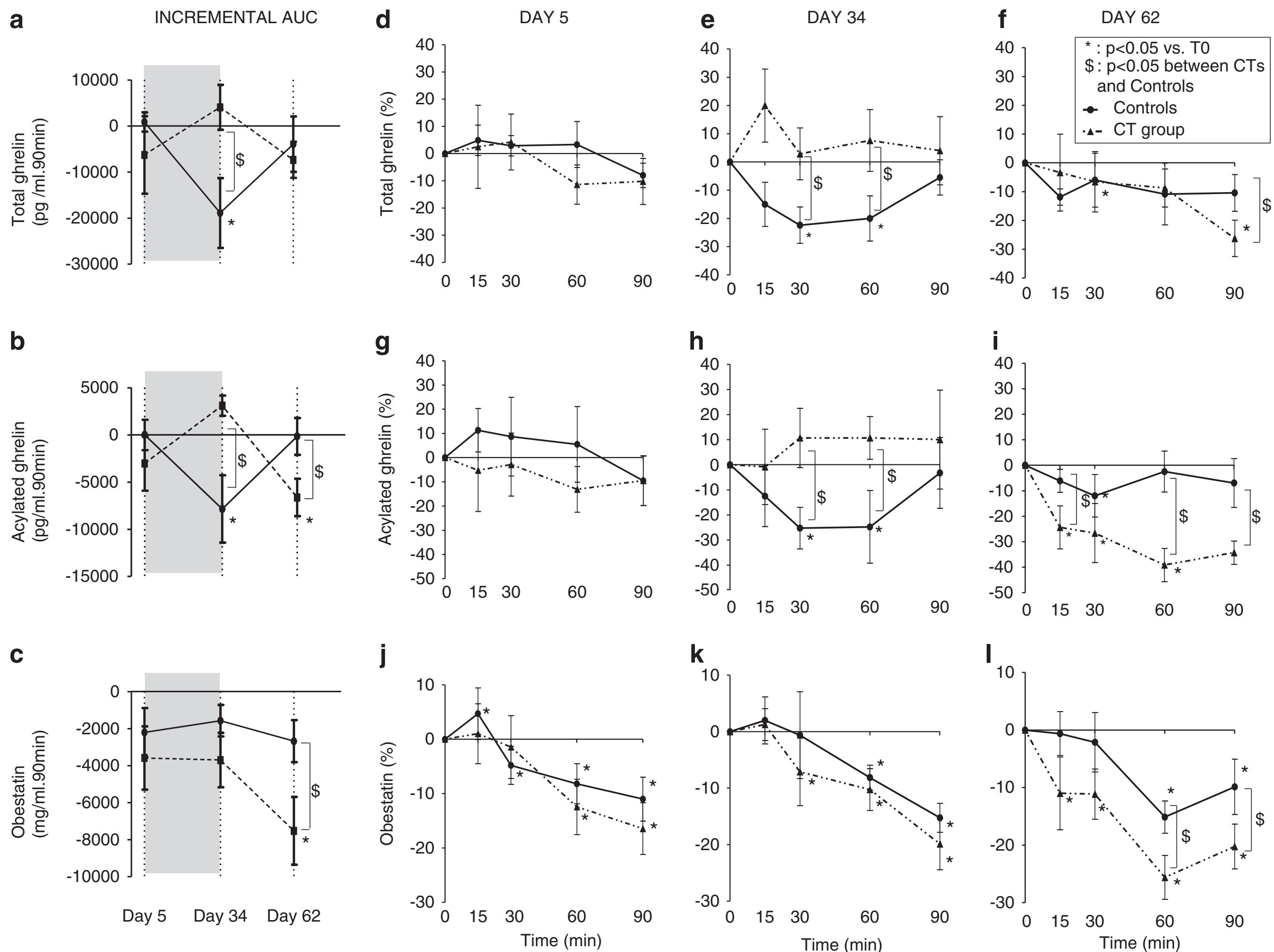

Figure 2. Ghrelin/obestatin post-test meal kinetic changes. Post-test meal kinetic changes in ghrelin/obestatin before overfeeding (day 5), at the end of the 2640 kJ (630 kcal) per day excess overfeeding period (day 34) and after *ad libitum* food intake period (day 62) in the CT group ($n = 8$, dotted line) and the control group (controls, $n = 8$, plain line). Standardized test meal (1373 kJ (328 kcal); 80% carbohydrates, 13% lipids and 7% proteins) was served and eaten between T0 min and 15 min. (**a**) iAUC for total ghrelin expressed as pg ml^{-1} per 90 min at each test meal (days 5, 34 and 62). (**b**) iAUC for acylated ghrelin expressed as pg ml^{-1} per 90 min at each test meal. (**c**) iAUC for obestatin expressed as mg ml^{-1} per 90 min at each test meal. (**d**) Kinetic changes in total ghrelin after test meal expressed as percentage change from baseline (T0) on days 5, (**e**) 34 and (**f**) 62. (**g**) Kinetic changes in acylated ghrelin after test meal on days 5, (**h**) 34 and (**i**) 62. (**j**) Kinetic changes in obestatin after test meal on days 5, (**k**) 34 and (**l**) 62. Data are expressed as mean ± s.e.m. Statistical analysis: *$P < 0.05$ vs T0 min in each group; $$P < 0.05$ between CTs and controls.

Indeed, overfeeding induced an earlier and longer surge of PYY and GLP-1 in CTs, associated with lower fasting ghrelin levels compared with controls. The post-prandial response in PYY and total GLP-1 in CTs could account for an earlier and stronger satiety signal. These data are consistent with CTs' dietary records revealing smaller-portioned meals and more snacking at baseline and after overfeeding. The link between snacking and bodyweight is not clear, recent data suggesting snacking is not necessarily associated with overweight.[33] This particular eating behavior could be adaptive in this thin population in order to ensure enough adequate daily energy intake. Secondly, CT could be considered as the opposite to obesity with regards to this particular satiety profile, as GLP-1 and PYY post meal responses are blunted in obese people[9,10] and orexigenic tone increases after diet-induced bodyweight loss.[8]

Ghrelin showed a physiological post-prandial fall[34] in controls, except for the first test meal, perhaps due to a possible stress at the first visit.[35] On the opposite, the fall of ghrelin was blunted in CTs before and after overfeeding. It could be surprising as post-prandial fall of ghrelin is blunted in obese patients.[36,37] The inability of food to suppress ghrelin in CTs after the overfeeding may be due to the already low fasting plasma level at that moment compared with controls, also accounting for this anorexigenic tone. It could also be due to an adaptive orexigenic reaction to the enhanced surge in PYY and GLP-1.

In obesity, some authors propose the adaptive orexigenic reaction during diet-induced weight loss as responsible for the relapse after restrictive diet.[8] By contrast, we hypothesize that enhanced anorexigenic/satietogenic tone found in CT after a supervised overfeeding could make them less prone to maintain longer overfeeding.

Importantly, CTs did not gain bodyweight after overfeeding. Moreover, the increase in oxygen consumption rate and the decrease in carbohydrate oxidation index accounted for a low fat storage in CTs (Table 1). This adaptive behavior contemporary to a lack of bodyweight gain markedly contrasts the expected increase in bodyweight and FM observed in controls, often called 'lean' in the literature.[13,15,38] In parallel, hierarchical clustering analysis analysis of metabolomics data revealed opposite changes in global clusters between the two groups of subjects (heatmap Figure 4b), suggesting the involvement of different/inverse metabolic pathways in response to the same dietary stress

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including mitochondria and FFA metabolism.[39] It could indeed suggest a diverse mitochondria metabolism in both groups in response to overfeeding, as phenylalanine, tyrosine and xanthurenic acid are differentially modified by overfeeding between the groups.[40] Moreover the opposite carnitine-level change in urinary metabolomics could suggest an opposite mitochondrial carnitine shuffle in response to overfeeding.[41]

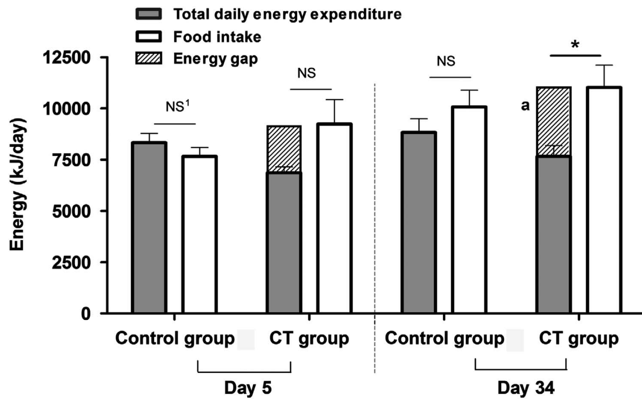

Figure 3. Energy balance. Energy balance before overfeeding (day 5), at the end of the 2640 kJ (630 kcal) per day excess overfeeding period (day 34) and after *ad libitum* food intake period (day 62) in the CT group ($n = 8$) and the control group (controls, $n = 8$). Total daily energy expenditure (grey bars) was calculated as follows: TEE = REE × physical activity level. The physical activity level was evaluated with an accelerometer. Food intake (white bars) was evaluated with a daily dietary record completed for 5 days. The energy gap (striped boxes) was calculated as follows: TEE − food intake. Data are expressed as mean ± s.e.m. Statistical analysis: *$P < 0.05$ between TEE and food intake. 1NS = non-significant. a indicates $P < 0.05$ vs day 5.

These metabolites could be considered as fingerprints of a different metabolomics pathway used by the groups.[42]

Bodyweight changes are related to energy imbalance. As a resistance to bodyweight gain was observed in CTs during overfeeding, one might presume an overreaction of energy expenditure mechanisms. This hypothesis is also supported by gut hormones profiles and changes in CTs. Thus, transgenic mice overexpressing PYY exposed to high-fat diet showed increased thermogenesis[43] and recent studies of infusion of PYY[44] and GLP-1 in humans showed increased REE.[45]

In the current study, the significant increase in CTs' REE observed during the overfeeding period can be proposed as an adaptive response to the fat overfeeding in order to maintain their low bodyweight. According to the literature, in basal conditions, REE in CTs, similar to that of controls, seems to be related to BAT FDG uptake.[46] Although difficult to isolate, the part of BAT activity within REE's rise in CTs after overfeeding remains to be studied.

However, despite the increase of REE, CTs had a positive energy balance by the end of the overfeeding period (that is, a higher food intake than TEE) while paradoxically not gaining bodyweight. Interestingly, a negative energy balance, the opposite to that seen in CTs, has been described previously in some obese patients, for whom, even after adjustment for underestimation of food intake and potential overestimated physical activity, the energy balance remained negative.[47] Further studies comparing opposite diet interventions (CTs compared with controls exposed to overfeeding/obese patients compared with controls exposed to food restriction) might be useful to compare these gaps. Altogether, these data support the hypothesis that CT's adaptive hormonal profile to overfeeding may select a particular energy expenditure pathway to prevent them from bodyweight gain. The '3500 kcal per pound' rule, recently questioned and debated,[48] cannot be applied to CT with regards to these data. This study provides another example of a specific population using a specific energy pathway, partly explaining conflicting interpretation of energy balance and needs therefore further studies to confirm it.

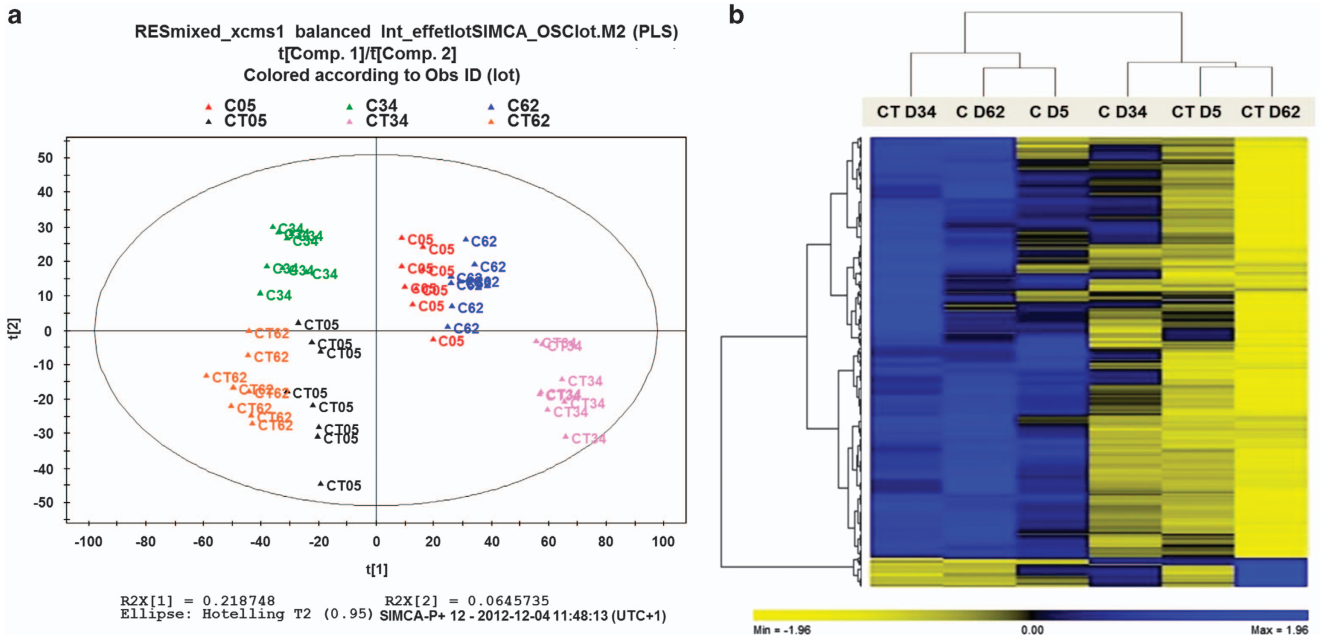

Figure 4. Metabolomics urine analysis. Metabolomics urine analysis before overfeeding (day 5), at the end of the 2640 kJ (630 kcal) per day excess overfeeding period (day 34) and after *ad libitum* food intake period (day 62) in the CT group ($n = 8$) and the control group (controls, $n = 8$). (**a**) PLS analysis with orthogonal signal correction (OSC-PLS) score plots of component 1 vs component 2 showing the group × time effect (CT34 R2cum = 0.62, CT34 Q2cum = 0.62). Day 5 controls are in red, day 34 controls in green, day 62 controls in blue, day 5 CTs in black, day 34 CTs in pink and day 62 CTs in orange. (**b**) Heatmap representing a hierarchical clustering analysis of the samples (group × time) and significant ions for interaction. The blue color corresponds to the highest value and yellow color to the lowest ones.

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The paradoxical gap between energy intake and energy expenditure found in CTs may raise questions on the accuracy of usual techniques of energy balance evaluation.[48] While AEE measured by accelerometer did not change after overfeeding, the increase of total energy expenditure in CTs might be specifically underestimated, at least in terms of non-exercise activity thermogenesis,[49] fidgeting[50] and/or subclinical steatorrhea due to fat overfeeding.[51] Further studies with calorimetric chambers or doubly labeled water tests could evaluate these parameters. Finally, CTs' compliance to the dietary protocol might be questioned, especially as there is no reliable method to measure energy intake in human. However, food questionnaires even if controversial are widely used. Besides, several indirect markers such as lipid plasma levels were modified by overfeeding in CTs. A metabolomics trajectory[52] was observed in both groups with significant changes in response to overfeeding (Figure 3a), suggesting that a dietary intervention was performed in both groups. Taken together, these data strongly suggest that CTs complied with the study protocol. Recent mathematical models proposed to predict weight gain/loss in response to dietary intervention[53] should be used to test compliance in further studies.

CONCLUSION

This study showed specific changes to a 4-week fat overfeeding in the particular CT population. The increase in the anorexigenic hormonal tone in response to the supervised overfeeding may partly prevent CTs from gaining bodyweight and may explain CTs' food behaviors. CTs failed to gain bodyweight and seem to use particular energy pathways. This study reveals that CTs exhibit a paradoxical positive energy balance, opposite to the paradoxical negative energy balance in obesity, suggesting a possible resistance to bodyweight gain in CT and to bodyweight loss in obesity. Understanding this energetic adaptation to overfeeding in CT could provide explanations on the mechanisms underlying resistance to bodyweight loss in obesity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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