A Novel Evaluation of 24-h Energy Metabolism in Cushing's Syndrome: The Metabolic Cost of Hypercortisolism

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Abstract

Context

Hypercortisolism leads to metabolic alterations (glucose dysregulation, increased protein catabolism, and increased lipolysis and lipogenesis) that contribute to weight gain, increased fat mass, and muscle loss in patients with Cushing's syndrome (CS).

Objective

We examined the relationship between body composition and 24-hour energy metabolism in patients with active disease.

Methods

This cross-sectional case-control study included 16 patients with active CS and 16 sex, weight and height-matched controls subjects.

Hormonal evaluation included serum cortisol, plasma ACTH, urinary free cortisol, 1-mg dexamethasone suppression test (DST) cortisol. Body composition was assessed by dual-energy X-ray absorptiometry. 24-hour energy expenditure (24hEE) and macronutrient oxidation rates were measured by 24-hour whole-room indirect calorimetry.

Results

Compared to control subjects, patients with CS exhibited higher total fat mass (+ 9 ± 14%, p=0.02) and trunk fat mass (+ 27% ± 21%, p<0.001), as well as reduced lean soft tissue (−7% ± 11%, p=0.02) and appendicular lean soft tissue (−14 % ± 14%, p<0.001). 24hEE was lower by −154 ± 275 kcal/d (p=0.04). Post-DST cortisol was positively correlated with protein oxidation rate (r=0.5, p=0.04) and with 24hEE (β = + 15.1 kcal/d per 1 µg/dL of post-DST cortisol, p=0.04) independently of lean soft tissue.

Conclusion

Using a whole-room indirect calorimetry, we have demonstrated that chronic active hypercortisolism in patients with Cushing's syndrome is associated with a reduction in 24hEE, likely driven by the progressive loss of lean soft tissue, due to sustained increase in protein oxidation.

Introduction

Patients with Cushing' syndrome (CS) have increased morbidity and mortality risk which persists even when appropriately treated [1, 2]. Most patients experience significant weight gain, and CS is recognized as a cause of secondary obesity [3, 4]. The increased mortality is primarily due to cardiovascular events, which are triggered by severe metabolic disorders resulting from excess glucocorticoids (GC) [5-

[8]. Hypercortisolism exerts detrimental effects on multiple organs, including the liver, muscle, adipose tissue, and pancreas [9]. These effects are primarily mediated by disruptions in glucose metabolism, such as enhanced gluconeogenesis and impaired insulin signaling leading to reduced glucose uptake and utilization, and dysregulated insulin secretion [10-12].

Excess GCs also play a crucial role in adipose tissue metabolism [13]. In white adipose tissue, GCs exert dual effects on lipid metabolism depending on the duration of exposure. While acute exposure (hours to days) promotes lipolysis [14], chronic exposure (weeks) leads to increased adiposity, particularly in visceral adipose tissue [15]. Hypercortisolism induces alterations in body composition, characterized by fat redistribution and preferential accumulation of central adipose tissue. Chronic hypercortisolism not only upregulates enzymes involved in *de novo* lipogenesis and fatty acid storage [16] but also impairs protein metabolism by reducing protein synthesis and enhancing proteolysis [17]. Consequently, the increased breakdown of proteins releases amino acids that serve as substrates for hepatic gluconeogenesis. Over time, this chronic GC-induced excessive degradation may contribute to skeletal muscle atrophy and loss [17, 18].

The combined effects of chronic GC excess may alter energy metabolism and macronutrients oxidation leading to increased fat mass and decreased lean body mass compared to healthy individuals. The aim of this study was to assess body composition, 24-hour energy expenditure (24hEE), and macronutrient (carbohydrate, lipid, and protein) oxidation rates in patients with active CS using the whole-room indirect calorimetry (metabolic chamber), the gold-standard method for assessing energy metabolism over a 24-hour period.

Materials and methods

Patients and Study design

This cross-sectional, 1:1 case-control study included 16 patients (11 women, 5 men) with endogenous, active CS. Patients were referred to the Endocrinology Unit (University Hospital of Pisa) by general practitioners or community outreach and underwent a series of assessments to establish or confirm the diagnosis. At the time of the study, all 16 patients had hypercortisolism: 14 were newly diagnosed and untreated, and 2 had persistent disease after transsphenoidal surgery. Of these, 9 patients were hospitalized, while the remaining 7 were evaluated on an outpatient basis. Baseline serum cortisol and plasma ACTH levels were measured at 08:00 AM. Before being enrolled in the study, an overnight 1-mg dexamethasone suppression test (DST) was performed to establish the diagnosis, as previously described [19]: Dexamethasone was administered orally between 23:00 and midnight (00:00 h), followed by a fasting blood sample collection between 08:00 and 09:00 the next morning to measure serum cortisol levels. Additionally, 24-hour urine samples were collected to assess basal urinary free cortisol (UFC), and cortisol rhythm was measured at 8:00 AM and midnight in the hospitalized patients (9/16). The diagnosis of endogenous CS was established based on well-defined clinical and laboratory criteria[20]. Twelve patients were diagnosed with ACTH-dependent CS of pituitary origin (Cushing's disease, CD: 10 micro- and 2 macro-adenoma). The remaining 4 had ACTH-independent CS, with 3 caused by adrenal adenomas (AA) and 1 by adrenal carcinoma (AC). In the latter case, the patient was untreated at the time of the metabolic chamber evaluation, having been referred for hypercortisolism assessment due to a suspected adrenal adenoma. Postoperative histology subsequently established the diagnosis of adrenocortical carcinoma. The demographic, anthropometric, clinical and laboratory data of patients are reported in **Table 1**. All patients had abnormal DST with cortisol levels exceeding 1.8 µg/dL (50 nmol/L) [20] and 9 patients who were hospitalized for the evaluation of associated comorbidities and complications had disrupted cortisol rhythm. Urinary free cortisol (UFC) levels were above the reference interval in 11

patients. All sixteen patients with active CS had at least one associated comorbidity, such as hypertension, type 2 diabetes, dyslipidemia and osteopenia/osteoporosis and were receiving specific treatment (**Table 1**). Among the 11 women, 3 were diagnosed with hypogonadism, presenting with amenorrhea for more than six months, 1 was in the menopausal stage, and 1 had both growth hormone (GH) deficiency and hypogonadism. In contrast, none of the 5 men had anterior pituitary defects. Three patients had secondary hypogonadism that could be attributed to suppression of the gonadal axis (**Table 1**). **Table 2** reports other relevant metabolic parameters, including blood pressure, fasting glucose, glycated hemoglobin (HbA1c), and thyroid function tests.

Sixteen control subjects matched for sex, weight (±5 kg) and height (±10 cm) were recruited as a control group. They were selected from healthy volunteers or from a previously characterized cohort of individuals with obesity who had been referred to our Center and showed no evidence of uncontrolled endocrine or metabolic disorders. All participants had previously provided informed consent and voluntarily agreed to undergo 24-hour energy metabolism assessments. The sixteen individuals best matched to the patient group based on sex, weight, and height were then selected as controls. In the control group, 10 subjects were not receiving any treatment, while the remaining participants were undergoing various therapies, including hypolipidemic therapy (n=2), vitamin D supplementation (n=2), a combination of antihypertensive and hypolipidemic therapy (n=1), and L-thyroxine therapy (n=1).

Both CS patients and control subjects were admitted to the whole-room indirect calorimeter (metabolic chamber) for a 24-hour assessment of energy expenditure and substrate oxidation while consuming a balanced isocaloric diet (50% carbohydrates, 30% fat, 20% protein). Meals were provided inside the chamber through an airlock at scheduled times: breakfast at 08:00, lunch at 12:00, and dinner at 19:00. The caloric content of the individualized diet was determined using standardized equation based on body weight, height, age and sex [21]. The proportion of total caloric intake provided at each meal was 20 % for

breakfast, 50 % for lunch, and 30 % for dinner. During their stay in the metabolic chamber, 24-hour urine was collected to assess UFC and nitrogen excretion. After exiting the metabolic chamber, a fasting measurement of body composition was performed by dual-energy X-ray absorptiometry (DXA) as described below. Body weight was measured using a digital electronic scale while participants wore light clothing. Standing height was measured to the nearest 1 cm with a stadiometer while participants were not wearing shoes. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (kg/m²). Overweight and obesity were classified according to established criteria [22].

The study was approved by the Regional Ethics Committee for Clinical Trials of the Tuscany, Region Section: AREA VASTA NORD OVEST (register number 19543). All patients gave their written and informed consent to participate. The procedures employed in the study were in accordance with the ethical standards of the Local Ethical Committee and with the Helsinki Declaration of 1964 and its later amendments.

Measurement of Body Composition

Body composition was assessed DXA Lunar Prodigy (GE Healthcare). The following parameters were measured [23]: Lean Soft Tissue (LST), defined as the estimated mass of all non-fat, non-bone mineral molecules in the body, including skeletal muscle mass, non-fat components of adipose tissue, skin, connective tissue, etc; Fat Mass (FM), the total mass of body fat; Appendicular Lean Soft Tissue (ALST), the sum of LST in the upper and lower extremities; Appendicular Fat Mass (AFM), the sum of FM in the upper and lower extremities; Trunk Fat Mass (TFM), defined as the difference between total FM and AFM.

Whole-room indirect calorimetry

Assessment of 24hEE and 24-hour respiratory exchange ratio (24hRER), an indicator of substrate oxidation, was performed using validated methods established in previous studies [24-26]. In brief, following

an overnight fast, participants entered the calorimeter at approximately 7:30 AM. Meals were provided at specific times (breakfast at 8:00 AM, lunch at 12:00 PM, and dinner at 7:00 PM), and any unconsumed food was weighed to accurately determine the actual 24-hour energy intake for each session. Both CS patients and control subjects remained in the calorimeter for 23.5 hours, during which time carbon dioxide (CO_2) production and oxygen (O_2) consumption were continuously recorded every minute. The 24hEE was then calculated using the Lusk formula [27]. The chamber air temperature was maintained at a constant 23.8 ± 1.4°C and the air inflow rate was controlled at a fixed 100 L/min using a mass flow controller. Air samples from both the inlet tube and the chamber were collected using membrane pumps, dried to a humidity level of <1000 ppm using a gas sample dryer driven by counterflow dry medical air, and then analyzed using absolute gas analyzers. Spontaneous physical activity was monitored using radar sensors and expressed as the percentage of time during which activity was detected [28]. The accuracy of the calorimeter system was periodically confirmed before and during the study period by burning instrument-grade propane on a calibrated scale each month, with recovery rates of oxygen (O_2) consumption, carbon dioxide (CO_2) production, and respiratory exchange ratio (RER) consistently within 5% of the values predicted by stoichiometry. Daytime energy expenditure in the inactive, awake state (EE_0) was determined as the intercept of the regression line between energy expenditure (EE) and spontaneous physical activity, measured at 1-minute intervals from 10:00 AM to 1:00 AM, and extrapolated to a 15-hour period [29]. Sleeping energy expenditure was calculated as the average energy expenditure recorded between 11:30 PM and 5:00 AM, excluding minutes with subject movement >1.5% (>0.9 seconds per minute). This value was then extrapolated to a 24-hour period (24h Sleeping Metabolic Rate, 24hSMR). Awake and fed thermogenesis (AFT) was calculated as the difference between EE_0 minus SMR (both expressed in kcal/min), and was extrapolated to the 15-hour awake period [29]. The 24hRER was calculated as the ratio of the 24-hour average CO_2 production to 24-hour average O_2 consumption. Carbohydrate (CarbOx) and lipid (FatOx) oxidation rates were derived from the nonprotein

24hRER and nonprotein O_2 consumption ($NPVO_2$) after adjusting for protein oxidation rate (ProtOx), which was estimated from 24-hour urinary nitrogen excretion [30], as follows:

$$\text{Carbohydrate oxidation rate (kcal/day)} = NPVO_2 \cdot [(NPRQ - 0.707)/0.293] \cdot 5.047$$

$$\text{Lipid oxidation rate (kcal/day)} = NPVO_2 \cdot [(1 - NPRQ)/0.293] \cdot 4.686$$

Nitrogen excretion was measured by using UREAL, Cobas c, Roche Diagnostics, Basel, Switzerland, with urinary nitrogen calculated from urea using the formula: urinary nitrogen = urea × 0.467.

Hormonal measurements

Serum cortisol levels were determined using a commercial chemiluminescence immunoassay (CLIA) kit (Cat# 33600, RRID:AB_2802133, Access Cortisol Immunoassay, Beckman Coulter Diagnostics, Beckman Coulter Brea, CA, USA), with a reference interval at 8:00 AM for normal subject of 6.7–22.6 mcg/dL. Plasma ACTH was analyzed using a chemiluminescence immunoassay (CLIA) (Cat# 0025221, RRID: AB_2783633, ST AIA-PACK ACTH, TOSOH Corporation, Tokyo, Japan,), with a reference interval at 8:00 AM for normal subject of7.2–63.5 pg/mL. The 24-hour UFC was quantified through high-performance liquid chromatography followed by mass spectrometry (LC-MS/MS) (EUROREK Srl., Chiaravalle, Italy), with a reference interval for normal subject of 11–70 mcg/24 hours. Thyrotropin (TSH), free triiodothyronine (FT3) and free thyroxine (FT4) were measured by a specific and sensitive automated chemiluminescent immunoassay (CLIA) (Cat# 191 2997, RRID:AB_3101993; Cat# 138 7000, RRID:AB_3101994, Vitros 3600 System, Ortho-Clinical Diagnostic, Rochester, NY, USA). The reference interval in euthyroid subjects was 0.4-4 mUI/L for TSH, 0.7-1.7 ng/L for FT4 and 2.7-5.7 ng/L for FT3. Total testosterone was measured by a specific and sensitive automated chemiluminescent immunoassay (CLIA) (Cat# 33560, RRID:AB_2905661, Access Testosterone assay Beckman Coulter, Brea, California, United States). The reference interval for serum testosterone was 1.75–7.8 µg/L in men and <0.98 µg/L in women. In two patients, testosterone measurements were not available.

Statistical analysis

All statistical analyses were performed using SAS, version 9.2 (SAS Institute, Cary, NC). The distribution of all main continuous variables was assessed using the Shapiro-Wilk test. All variables included in the main analyses did not significantly deviate from normality and were therefore reported as mean ± standard deviation (SD) and analyzed using parametric methods P-values <0.05 were considered statistically significant. To account for multiple testing in the between-group comparisons, we performed a post hoc Holm-Bonferroni correction across the prespecified outcomes in Tables 3 and 4, using a family-wise alpha of 0.05.

Differences in anthropometric, hormonal and metabolic measures between groups were evaluated using Student's paired t-test. The Pearson correlation index was used to quantify the relationships between hormone concentrations and energy metabolism measurements (24hEE and its components) in the CS group. Multivariable regression analysis was performed to assess the associations between hormone concentrations and 24hEE adjusting for their known determinants (i.e., age, LST, and FM).

Although control subjects were closely matched to patients for weight, height, and sex, a small difference in mean age persisted. Accordingly, we performed additional analyses using analysis of covariance (ANCOVA) with age as a covariate. The primary results remained materially unchanged after this adjustment, suggesting that observed group differences were not attributable to age.

Results

Demographic, anthropometric and body composition parameters in patients with active, endogenous CS compared with control subjects are shown in **Table 3**.

Assessment of body composition

Compared to control subjects who were rigorously matched for weight and height (**Fig. 1A, 1B**), patients with CS exhibited a significantly higher FM (+3.5 ± 5.3 kg, p=0.02, **Figure 1C**) and TFM (+5.3

± 4.1 kg, p<0.001, **Figure 1D**) while displaying a lower LST (−3.5 ± 5.5 kg, p=0.02, **Figure 1E**) and ALST (−3.3 ± 3.4 kg, p<0.001, **Figure 1F**).

24hEE and macronutrients oxidation

As shown in **Table 4 and Figure 2**, patients with CS exhibited lower 24hEE (−154 ± 275.1 kcal/d, p=0.04 **Figure 2A**) and 24hSMR (−146 ± 273.0 kcal/d, p=0.04 **Figure 2B**), likely attributable to a reduction in fat oxidation. In contrast, protein oxidation did not differ significantly, despite lower LST in patients with CS, and neither did EE_0 and AFT (**Table 4**). After applying the Holm-Bonferroni correction for multiple tests, the comparisons that were nominally significant in the unadjusted analyses lost statistical significance (all adj. p>0.05). **Figure 3** presents the average minute-by-minute average 24hEE trajectories over the 24-hour period in patients with CS and control subjects.

To investigate the potential role of hypercortisolism in the previously mentioned findings, we examined the impact of biochemical markers of hypercortisolism on 24-hour energy metabolism. Associations were first evaluated without adjustment and then using multivariable regression models. Notably, in patients with CS, a significant positive association was observed between post-DST cortisol and 24hEE (**Figure 4A**). In a multivariable model adjusting for known determinants of 24hEE (LST, FM, and age), only LST (β = + 17.3 kcal/d per 1 kg of LST, p=0.012) and post-DST cortisol (β = + 15.1 kcal/d per 1 µg/dL of post-DST cortisol, p=0.04) remained positively and independently associated with 24hEE, indicating that both LST and cortisol contribute to variation in EE.

Furthermore, post-DST cortisol positively correlated with ProtOx (**Figure 4B**), with no significant association with CarbOx or LipOx (all p > 0.1). Of note, ProtOx in CS patients was not associated with demographic or anthropometric measures such as age, LST and FM (all p>0.2).

Although post-DST cortisol was positively associated with UFC (r= 0.72, p=0.002) and with midnight cortisol at 24 AM (r= 0.7, p=0.03), no significant associations were observed between UFC or midnight cortisol (n=9) and 24hEE or its components (all p>0.1).

Discussion

This study is the first to simultaneously assess 24h energy metabolism using a whole-room indirect calorimetry and body composition using DXA in patients with CS. Additionally, we evaluated a group of closely matched control subjects to provide a comparative analysis.

Our findings confirm that, compared to weight-matched control subjects, patients with hypercortisolism exhibit an increased fat mass and trunk fat mass along with a marked decrease in lean soft tissue, consistent with the characteristic clinical phenotype [31-35]. We also observed lower 24hEE and 24hSMR in individuals with CS, along with a significant association between energy expenditure and cortisol concentrations following the dexamethasone suppression test.

To our knowledge, only one previous study has concurrently assessed the impact of hypercortisolism on both body composition and energy metabolism in patients with endogenous CS[36], using an open-circuit ventilated hood system. In this cohort of 18 patients with CS, total lean mass was approximately 15% lower, particularly in the appendicular region, while total fat mass was about 30% higher, predominantly in the trunk, compared to control subjects. However, resting energy expenditure and macronutrient oxidation did not differ significantly, likely due to methodological differences, as the ventilated hood system lacks the precision needed for 24hEE measurements. In addition, its ability to assess macronutrient oxidation is limited, particularly for protein oxidation, which is best determined through 24-hour urinary nitrogen excretion. In the present study, with equivalent lipid and protein intake in patients with CS and matched control subjects, 24hEE and its component measurements suggest that hypercortisolism may lead to the observed changes in body composition through reduced fat oxidation. Protein oxidation did not differ between groups despite LST in CS and was associated with the degree of hypercortisolemia following the dexamethasone suppression test.

The potential relationship between biochemical markers of hypercortisolism and distinct macronutrient oxidation patterns is complex. Our findings indicate that elevated cortisol levels post DST are correlated with increased 24hEE and enhanced protein oxidation, independently of LST. This is in line with previous studies both in healthy volunteers and patients with CS. An acute hydrocortisone infusion in 9 healthy subjects led to a 9–15% increase in resting EE, along with enhanced alanine turnover and synthesis [37]. In 7 healthy adults, compared to saline infusion, an 8-hour infusion of hydrocortisone resulted in a significant increase in the rate of leucine appearance [38]. Dexamethasone treatment in 14 healthy volunteers also led to a 40% increase in plasma alanine concentration. Similarly, in 4 patients with CS, alanine levels were 40% higher compared to normal control subjects [39]. In healthy individuals, a 5-day administration of dexamethasone (8 mg/day) led to a negative nitrogen balance, driven by a persistent and early rise in urea nitrogen excretion [40]. In 20 healthy male volunteers, both intravenous infusion and 4-day oral methylprednisolone therapy led to increased EE and protein oxidation compared to placebo [41]. Taken together, these findings suggest that over time the increase in EE due to increased cortisol exposure, coupled with enhanced protein oxidation, may contribute to progressive muscle loss in individuals with chronic hypercortisolism. The consequent decline in LST, along with reduced fat oxidation, may ultimately lead to a reduction in 24hEE.

Although the etiology of CS vary across its different forms, the DST is most frequently used for screening and diagnosis for hypercortisolism[20] with various reported rates of false positive and false negatives; however it appears to be the most sensitive test in adrenal CS, including MACS [42].

Importantly, the 1-mg DST cortisol should be interpreted as a continuum [43] as higher post-DST cortisol levels have been associated with an increased risk of morbidity and mortality in large-scale studies [44-47]. In this context, a clinical and biochemical scoring system which included post-DST cortisol was recently

used to assess the severity of hypercortisolism in both ACTH-dependent and independent hypercortisolism [48].

The reliance on BMI as a sole measure of obesity has been reconsidered, emphasizing the importance of body composition assessment in accurately evaluating health risks across all populations [49, 50]. As previously mentioned, CS is a well-recognized cause of significant central obesity which increases the risk of cardiovascular disorders and potentially also the risk of cancer [51]. Understanding the causes of obesity in CS is crucial, as this phenotype has primarily been attributed to increased energy intake driven by cortisol excess and abnormal fat redistribution [41, 52]. However, findings from the current study suggest that a reduction in EE may also contribute to body fat accumulation.

Our cross-sectional study, which included matched control subjects for patients with CS, assessed 24hEE and its components measured by indirect whole-room calorimetry, a method that provides valuable advantages over other EE measurement techniques. The precise matching of participants strengthens the validity of the observed body composition differences by minimizing potential confounding due to overall body size. However, some limitations should be acknowledged. Our limited sample size may have somewhat reduced our ability to detect subtle differences in macronutrient oxidation. Due to the rarity of Cushing's syndrome, recruiting a larger cohort within a single center over a limited period was challenging, and selecting control patients with obesity, carefully matched for sex, weight, and height, was equally difficult. Thus, no formal power calculation was performed prior to study initiation. Given the small sample size and the fact that many of the variables analyzed are correlated or derived from each other, adjustment for multiple tests using the Holm Bonferroni procedure eliminated statistical significance of the nominal findings. Because this was an exploratory and hypothesis-generating study in a rare-disease cohort, these results should be interpreted as preliminary and with caution, and confirmation in larger independent cohorts will be important for

future research. Furthermore, the cross-sectional design restricts our capacity to draw causal inferences regarding the impact of hypercortisolism on energy metabolism. In this regard it will be important to verify whether the observed alterations in body composition and energy metabolism persist after correction of hypercortisolism. It should also be noted that in patients with Cushing's syndrome, central hypogonadism is frequently reported due to hypercortisolism induced suppression of the gonadal axis [53-55], and this condition might contribute to the development of sarcopenia. Additionally, partial aldosterone co-secretion might contribute to influence thermoregulation. Another limitation of our study is the lack of insulin measurements, which precluded calculation of the HOMA index. Although our study focused on patients with endogenous Cushing's syndrome, it is plausible that similar alterations in energy metabolism may occur in patients with exogenous hypercortisolism. Given the higher prevalence of the latter, future studies are warranted to investigate whether these findings can be extended to that population, potentially broadening the clinical impact of our results.

In conclusion, the findings of our study suggest that chronic hypercortisolism is associated with a reduction in 24hEE, presumably driven by the progressive loss of LST. The degree of hypercortisolism, as assessed by the DST was associated with protein oxidation, suggesting a potential etiologic role in the loss of LST. These metabolic alterations ultimately contribute to central obesity and to the characteristic features of a cushingoid phenotype. The presence of sarcopenic obesity should prompt clinical suspicion of underlying endogenous hypercortisolism, even in the absence of overt Cushingoid features. Our results further highlight the potential importance of recognizing and treating mild hypercortisolism at an early stage, to prevent the development or progression of sarcopenia with consequent reduction in energy expenditure.

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Author Contributions

A.B., I.L. and F.S. designed the study protocol, interpreted data and wrote the manuscript.

R.J., and V.A. performed nutritional counseling.

P.P. and A.L. managed the engineering procedures and carried out data extraction from the metabolic chamber.

C.B., C.U., L.M., G.S., P.F. conducted the recruitment of patients and controls.

E.V. performed the evaluation and interpretation of the DXA scans.

L.C., J.K., and M.F. assisted with the interpretation of the data, literature review and revised the manuscript.

All authors read, critically revised the draft and approved the final manuscript. A.B. and F.S. have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Data availability:

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Figures legends

Figure 1. Body composition measures in patients with Cushing's syndrome (CS) and control subjects (CON): Body weight (A), BMI (B), fat mass (C), trunk fat mass (D), lean soft tissue (E), and appendicular lean soft tissue (F).
Error bars represent mean ± SD.
*: $p<0.05$ by Student's paired t-test.
Abbreviations: BW: Body weight, BMI: body mass index, FM: fat mass, TFM: trunk fat mass, LST: lean soft tissue, ALST: appendicular lean soft tissue.

Figure 2. Energy metabolism in patients with Cushing's syndrome (CS) and control subjects (CON): 24hEE (A) and 24hSMR (B).
Error bars represent mean ± SD.
*: $p<0.05$ by Student's paired t-test.

Figure 3. Energy expenditure (EE), measured over 24 hours using a whole-room indirect calorimeter in patients with Cushing's syndrome (CS, red line) and control subjects (CON, black line). The graph illustrates the average EE trajectories recorded every minute (kcal/min)
The time frame between 7:30 and 9:30 AM was omitted from the graph because, upon entering the chamber, participants initiated the urine collection, consumed breakfast, and changed into appropriate clothing.

Figure 4. Relationships between post-DST cortisol and 24hEE (A) and ProtOX (B). Pearson correlation coefficient (r) is reported along with its' significance (p).

Table 1. Demographic, anthropometric, clinical and laboratory data of patients with active Cushing's syndrome.

Patients	*Sex*	*Age*	*BMI*	*Origin*	*Comorbidities*	*Comorbidities treated*	*ACTH at 8:00 (ng/L)*	*Cortisol at 8:00 (µg/dL)*	*Cortisol at 24:00 (µg/dL)*	*Post-DST cortisol (µg/dL)*	*UFC (µg /24h)*	*Tropin defects*	*Testosterone (µg/L)*
1	F	27	25.7	Pm	HypoT, DLP	HypoT	95	13	-	16	616	LH/FSH	0.4
2	F	53	30.6	Pm	HTN, DLP	HTN, DLP	82	13.8	11.8	2.4	86	GH, LH/FSH	0.5
3	M	74	29.8	AA	HTN, DLP	HTN, DLP	2	15.1	22.2	15.9	180	LH	1.3
4	M	37	42.4	AA	HTN, DLP	HTN, DLP	2	17.9	21.1	23.3	463	LH	0.7
5	M	63	27.3	Pm	DLP	DLP	35	8.5	7.9	9.1	40	no	4.8
6	M	62	28.8	AC	HTN, DLP, T2D	HTN	3	19.6	-	20.4	342	LH	0.8
7	F	55	34.8	AA	HTN	HTN	16	13.5	-	3.7	84	no	NA
8	F	32	32.2	PM	HTN	HTN	98	16.2	-	14.4	284	no	0.9
9	F	32	42.4	Pm	HTN	HTN	129	19.5	-	10.3	46	no	NA
10	F	50	23.3	Pm	HTN, DLP, T2D, HypoT	HTN, DLP, HypoT	37	15.4	-	17.8	370	no	0.2
11	F	42	20.6	PM	HTN, DLP, OP	HTN, DLP, OP	49	7.2	-	3.2	38	no	0.3
12	F	45	45.9	Pm	HTN, DLP, HypoT	DLP, HypoT	20	13.4	12.4	7.5	52	no	0.5
13	F	53	31.0	Pm	DLP	DLP	43	18.2	23.5	18	588	LH/FSH	0.5
14	M	49	36.9	Pm	HTN, DLP, T2D, HypoT	HTN, DLP, T2D, HypoT	22	24.3	20.9	24.7	419	no	2.7
15	F	57	31.6	Pm	HTN, DLP, T2D, OP	HTN, DLP, T2D, OP	27	14.4	10.8	2.9	37	no	0.5

16	F	54	30.6	Pm	HTN, DLP, T2D	HTN, DLP, T2D	80	14.6	9.3	20.3	99	LH/FSH	0.3
Mean±SD	-	49±12	32.1±7.0	-	-	-	46.7±38.7	15.3±4.2	15.5±6.2	13.2±7.6	234.0±206.5	-	1.03±1.3

Normal reference ranges in healthy subjects are: serum cortisol at 8:00 AM, 6.7–22.6 µg/dL; serum ACTH at 8:00 AM, 7.2–63.5 pg/mL; and 24-hour urinary free cortisol (UFC), 11–70 µg/24 h. The reference interval for serum testosterone was 1.75–7.8 µg/L in men and <0.98 µg/L in women. In two patients, testosterone measurements were not available. Midnight cortisol was not available for the 7 patients evaluated in an outpatient setting. Data are also reported as mean ± standard deviation.

Abbreviations: AA: adrenal adenomas; AC: adrenal carcinoma; hypoT: hypothyroidism; HTN: hypertension; DLP: Dyslipidemia; OP: osteopenia; Pm: pituitary microadenomas; PM: pituitary macro-adenomas; T2D Type 2 Diabetes.

Table 2. Metabolic parameters of patients with active Cushing's syndrome.

Patients	*Blood pressure (mmHg)*	*Glycated hemoglobin (HbA1c, %)*	*Fasting glucose (mg/dL)*	*TSH (mUI/L)*	*FT4 (ng/dL)*	*FT3 (ng/L)*
1	115/70	5.9	105	2.2	0.7	2.7
2	150/80	5.4	95	1.8	3.4	0.7
3	140/90	5.5	80	2.0	1.0	3.1
4	123/70	5.9	84	0.5	0.7	2.9
5	124/69	5.4	101	2.6	0.8	3.4
6	130/80	6.9	105	0.3	1.1	3.5
7	121/80	5.6	74	1.9	1.1	3.8
8	140/90	5.9	78	0.7	0.6	3.2
9	130/95	5.8	84	0.7	0.6	3.4
10	140/88	5.6	74	0.6	1.3	3.4
11	118/74	6.0	78	0.8	1.1	4.0
12	128/88	6.1	97	1.4	1.1	3.4
13	135/78	5.2	88	0.5	1.0	3.0
14	136/93	5.6	89	1.4	0.8	2.7
15	130/84	6.3	90	0.4	0.9	3.2
16	143/96	9.9	108	2.4	0.8	3.2
Mean±SD	130/83	6.0±1.1	89±11.3	1.3±0.8	1.1±0.7	3.1±0.7

Table 3. Demographic and body composition data of patients with CS and CON

	CS (n=16)	CON (n=16)
Sex (F/M)	11/5	11/5
Age (years)	49 ± 13	45 ± 14
Body weight (kg)	88.5 ± 20.8	88.6 ± 21.1
BMI (kg/m^2)	32.1 ± 7.0	32.3 ± 7.8
FM (kg)	40.7 ± 14.2 *	37.2 ± 14.3
TFM (kg)	24.7 ± 9.6 *	19.4 ± 8.4
AFM (kg)	14.9 ± 5.4	16.8 ± 6.3
LST (kg)	45.5 ± 9.7 *	48.9 ± 11.1
ALST (kg)	20.2 ± 4.9 *	23.5 ± 5.6

Each parameter is expressed as mean ± SD.
*: $p<0.05$ by Student's paired t-test.
Abbreviations: AFM: appendicular fat mass; ALST: appendicular lean mass; BMI: body mass index; CON: control subjects; CS: Cushing Syndrome's patients; F: females; FM: fat mass; LST: lean soft tissue; M: males; TFM: trunk fat mass.

Table 4. **Dietary intake and metabolic parameters of patients with CS and CON.**

	CS (n=16)	CON (n=16)
Dietary intake		
Prescribed total intake (kcal/d)	1738.8 ± 298.2	1795.6 ± 292.3
Actual total intake (kcal/d)	1677.8 ± 315.6	1742.4 ± 295.2
CHO intake (kcal/d)	888.0 ± 197.8	943.7 ± 94.9
Fat intake (kcal/d)	512.0 ± 89.3	522.6 ± 55.1
PRO intake (kcal/d)	333.0 ± 55.5	335.0 ± 55.1
Metabolic measures		
24hEE (Kcal/d)	1685.3 ± 300.0 *	1839.8 ± 449.4

24hSMR (Kcal/d)	1248.0 ± 284.8 *	1394.0 ± 428.5
EE_0 (kcal/15h)	1102.0 ± 192.0	1195.1 ± 278.1
AFT (kcal/15h)	365.0 ± 131.2	370.0 ± 108.2
CarbOx (Kcal/d)	691.9 ± 234.2	748.7 ± 321.0
FatOx (Kcal/d)	646.3 ± 314.8	790.5 ± 427.2
ProtOx (Kcal/d)	325.2 ± 62.3	303.3 ± 88.8

Each parameter is expressed as mean ± standard deviation.
Prescribed total energy intake (kcal/d) refers to the amount of energy allocated to patients and control subjects in the metabolic chamber and the actual total energy intake (kcal/d) represents the energy effectively consumed, accounting for any unconsumed food during the session in the metabolic chamber.
*: $p<0.05$ by Student's paired t-test.
Abbreviations: CarbOx: carbohydrate oxidation; CHO: carbohydrate; CON: control subjects; CS: Cushing Syndrome's patients; CON: control subjects; FatOx: fat oxidation; PRO: protein; ProtOx: protein oxidation.

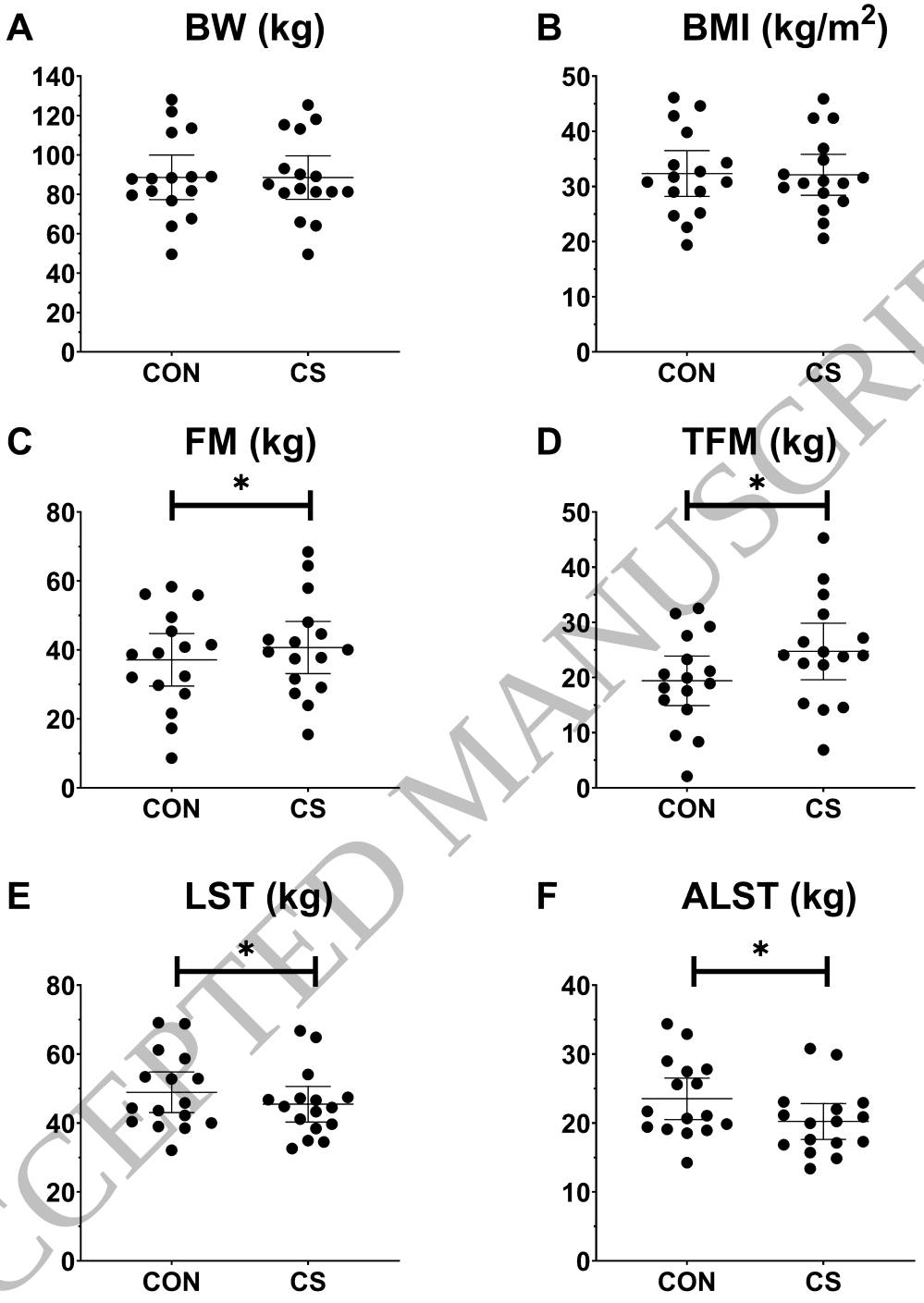

Figure 1
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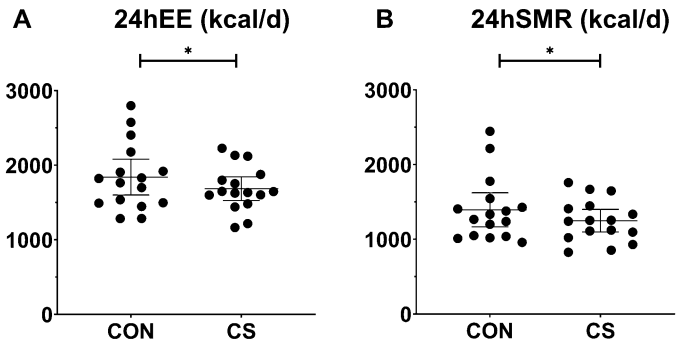

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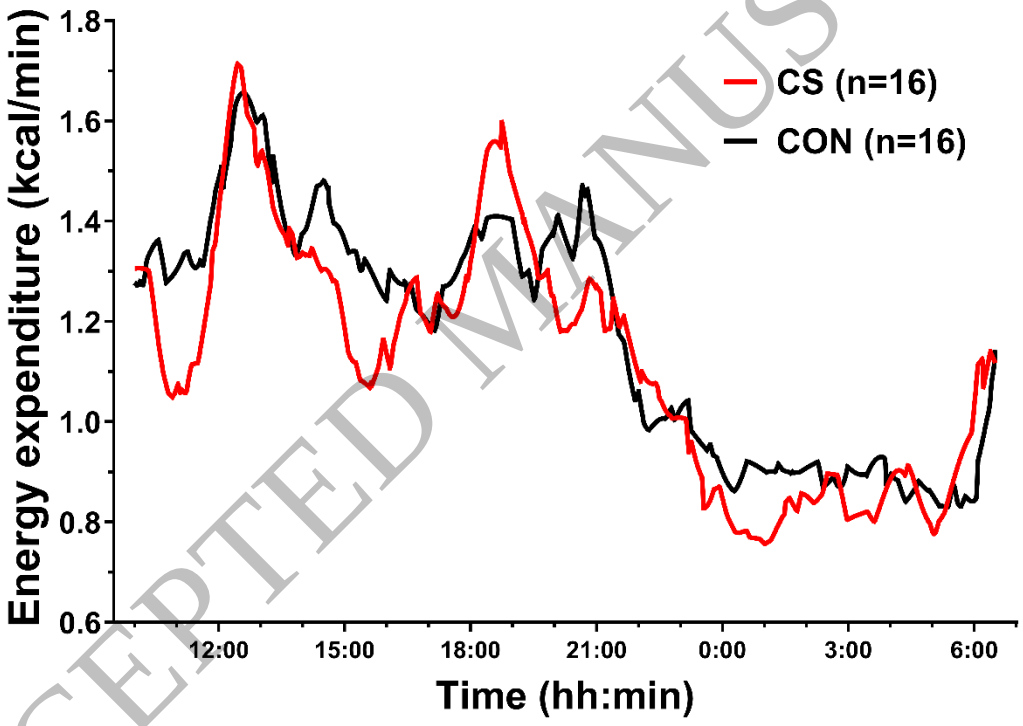

Figure 3
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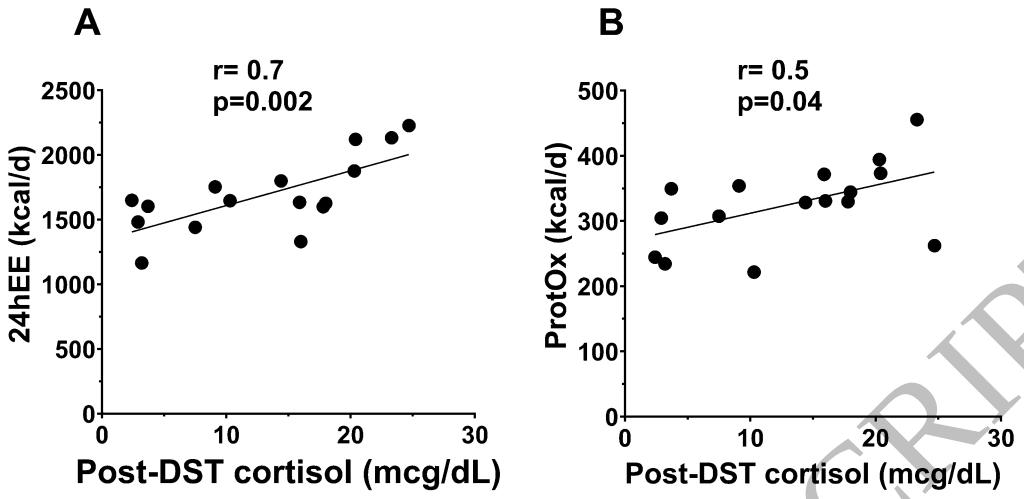

Figure 4
147x71 mm (DPI)