Evidence of Persistent Mild Hypercortisolism in Patients Medically Treated for Cushing Disease: the Haircush Study

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Abstract

Context: Cortisol-lowering drugs may not restore a normal cortisol secretion in Cushing disease (CD).

Objective: This work aimed to assess the long-term cortisol exposure in medically treated CD patients using hair-cortisol (HF) and hair-cortisone (HE) measurement.

Methods: This multicenter prospective study included 3 groups of female patients: CushMed = 16 treated with a stable cortisol-lowering drug dosage and normal urinary free cortisol (UFC); CushSurg = 13 cured by pituitary surgery; CushBla = 15 receiving stable recommended doses of hydrocortisone following bilateral adrenalectomy. Patients were evaluated for 3 months with their usual treatments. Two late-night saliva and 24-hour urine samples were collected monthly in CushMed, and at study end in CushSurg and CushBla patients. A 3-cm hair sample was collected at study end from all patients. Main outcome measures included clinical score and centralized measurement of UFC, late-night salivary cortisol (LNSF), late-night salivary cortisone (LNSE), HE, HF.

Results: Despite having almost all UFCs normalized, CushMed patients exhibited increased HE as compared to CushSurg controls ($P = .003$). CushMed patients also had increased clinical score ($P = .001$), UFC ($P = .03$), LNSF, LNSE ($P = .0001$), and variability in the latter parameters ($P = .004$). CushBla patients had increased HF and HE, contrasting with LNSEs similar to CushSurg patients. Six of 15 CushMed patients exhibited increased HE concentrations and had increased antihypertensive drug dosage compared to CushMed patients with normal HE ($P = .05$).

Conclusion: Despite normalized UFCs, a subset of medically treated CD patients displays an altered circadian rhythm of serum cortisol. A single HE measurement identifies chronic mild persistent hypercortisolism and could replace multiple saliva analyzes to monitor medical treatments in CD patients once UFC is normalized.

Key Words: Cushing disease, hair cortisol, hair cortisone, pharmacological treatment, bilateral adrenalectomy, late-night salivary cortisone

Abbreviations: BLA, bilateral adrenalectomy; BMI, body mass index; CD, Cushing disease; CushMed:, patients with CD treated with cortisol-lowering drugs; CushSurg:, patients in remission of CD following pituitary surgery; CushBla:, patients with CD treated by bilateral adrenalectomy; CV, coefficient of variation; HE, hair-cortisone; HF, hair-cortisol; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LNSE, late-night salivary cortisone; LNSF, late-night salivary cortisol; UFC, urinary free cortisol.

Cushing disease (CD) is a severe condition responsible for multiple comorbidities and increased mortality (1). Effective treatment is essential to improve comorbidities and long-term quality of life, and to reduce mortality. Surgical excision of the pituitary corticotroph adenoma is the ideal and first-line treatment of CD. However, even when performed by expert neurosurgeons, surgery is unsuccessful in 15% to 25% of patients and recurrence of the disease after a transient remission is observed in 15% to 25% of patients (1). Medical treatments targeting the pituitary adenoma to decrease adrenocorticotropin production and adrenal steroidogenesis inhibitors to decrease cortisol production are commonly used when surgical

Table 1. Main characteristics of participants according to study group

Patient group	CushMed	CushSurg	CushBla	*P*
No. of patients	16	13	15	
Age, y	52.8 ± 13.4	44.1 ± 12.8	49.3 ± 12.8	.21
BMI	30.4 ± 2.3	25.5 ± 1.4	29.4 ± 1.6	.17
Prior pituitary surgery	14	13	14	NA
Prior pituitary radiotherapy	3	0	5	NA
Duration of "control of hypercortisolism" period, mo	23.5 (15.0-69.7)	42.0 (13.0-53.0)	72.0 (43.0-106.0)[a,b]	.01
Clinical score	5.0 (3.0-6.0)	1.0 (0.0-1.0)[a]	2.0 (1.0-4.0)[a]	.0001
Urinary free cortisol, µg/24 h	28.1 (17.1-41.8)	21.6 (14.9-27.2)[a]	29.6 (21.1-52.4)[b]	.03
Late-night salivary cortisol, nmol/L	2.32 (1.36-3.43)	0.66 (0.44-1.08)[a]	1.10 (0.56-2.54)[a,b]	<.0001
Late-night salivary cortisone, nmol/L	16.0 (10.2-21.2)	5.0 (3.6-6.7)[a]	2.9 (1.5-6.8)[a]	<.0001
Hair cortisol, pg/mg	6.3 (3.0-26.1)	3.5 (3.1-5.3)	16.9 (8.4-46.7)[a,b]	.002
Hair cortisone, pg/mg	26.8 (17.6-72.9)	13.6 (10.8-20.3)[a]	27.7 (19.5-56.7)[b]	.003
Hair cortisol/cortisone ratio	0.23 (0.17-0.33)	0.25 (0.18-0.29)	0.54 (0.40-0.99)[a,b]	.0002

Categorical variables expressed as number. Continuous variables expressed as mean ± SD or median (25th-75th percentiles) for those with skewed distribution. The upper limit of the normal range for urinary free cortisol is (66 µg/24 h). Comparisons performed using χ^2, analysis of variance, or Kruskal-Wallis tests. *P* less than .05 was statistically significant.
Abbreviations: BMI, body mass index; CD, Cushing disease; CushMed: patients with CD treated with cortisol-lowering drugs; CushSurg: patients in remission of CD following pituitary surgery; CushBla: patients with CD treated by bilateral adrenalectomy; NA, not available.
[a]Post hoc pair-comparisons were performed to detect statistically significant difference vs group 1.
[b]Vs group 2.

treatment is ineffective or impossible (1, 2). The most common use of these drugs is a titration strategy including adaptation of drug dosage to normalize urinary free cortisol (UFC) while avoiding adrenal insufficiency (3). However, the use of UFC for this purpose has some limitations including difficulties in obtaining a complete 24-hour urine collection, and the fact that UFC assesses cortisol production only over a 24-hour period in a disease characterized by highly variable intensity of hypercortisolism (4). A few studies using late-night salivary cortisol (LNSF) measurement found persistent nocturnal mild hypercortisolism in a subset of CD patients despite normalization of UFC with cortisol-lowering drugs (5–9). LNSF measurement may therefore be useful to identify mild hypercortisolism (10, 11) and help to adapt cortisol-lowering treatments (1, 3, 6). However, LNSF also measures cortisol only at a specific point, and therefore must be repeated to ensure a valid assessment.

Measurement of hair cortisol (HF) and cortisone (HE) concentrations is a noninvasive method to assess the cumulative exposure of tissues to cortisol over much longer periods than is possible with assessments in blood, saliva, or urine. Measurements of HF and HE in a single scalp hair sample has a diagnostic accuracy for overt Cushing syndrome similar to currently used tests (12–14). Furthermore, recent studies have shown that it may also be useful to diagnose mild cortisol excess (14). To date, no data are available concerning HF and HE in comparison with commonly used biological tools to monitor cortisol-lowering medical treatments in patients with CD.

The purpose of our study was to take advantage of the ability of HF and HE measurement to assess long-term cortisol exposure in patients with CD receiving medical therapy. Specifically, we made the hypothesis that a subset of patients with pharmacological control of UFC may still exhibit mild hypercortisolism that can be identified by HF and HE measurement. To test this hypothesis, we conducted a study comparing HF and HE measurement to that of multiple measurements of UFC, LNSF, and late-night salivary cortisone (LNSE) in patients considered as medically "controlled" based on normalization of UFC measurements and in patients cured by pituitary surgery and bilateral adrenalectomy (BLA).

Methods

Participants

Three groups of women with a history of CD were studied (Table 1). The diagnosis of CD was based on standard cri- teria including identification of an adrenocorticotropin-immunostaining adenoma following pituitary surgery, occurrence of corticotropic insufficiency after pituitary surgery, or the results of bilateral simultaneous inferior petrosal sinus sampling.

The CushMed group consisted of patients with hypercortisolism treated with cortisol-lowering drugs secondary to failure or contraindication to surgery. Only patients treated using a titration regimen aiming to control UFC were in- cluded. Patients receiving an association with glucocorticoids and those treated with mitotane were excluded. To be included, patients had to be treated for at least 3 months with a stable drug dosage and having hypercortisolism "con- trolled" based on at least 2 normal UFCs during this period. Two control groups were used: CushSurg and CushBla. CushSurg consisted of patients in remission following pituit- ary surgery for at least 1 year with a normal biological evalu- ation of cortisol secretion during the 3 months preceding the start of the study. Biological evaluation consisted of a normal 24-hour UFC associated with a normal overnight dexamethasone suppression test (cortisolemia post test <50 nmol/L), or a normal midnight serum cortisol, or a normal LNSF according to the normal range of the local laboratory. Partial corticotropic insufficiency was ruled out in all CushSurg patients based on an early-morning serum cortisol concentration greater than 400 nmol/L or, for patients whose serum cortisol was within the normal test range but below this

threshold, by a positive response to the insulin tolerance test or the short synacthen test. CushBla consisted of patients treated by BLA. To be included, patients had to be treated with stable and recommended doses of hydrocortisone (eg, 15-25 mg/d) (15, 16) for at least 3 months before the study, and the last daily dose of hydrocortisone should have been administered no later than 5 PM.

Exclusion criteria for the whole study were use of topical or systemic corticosteroids (with the exception of hydrocortisone replacement in CushBla), kidney failure, poor compliance, hair length <3 cm, uncontrolled depression, drug addiction and alcoholism, myocardial infarction or stroke in the preceding 3 months, intense physical exercise, night workers, and patients with uncontrolled diabetes (glycated hemoglobin A_{1c} >9%) or suffering from frequent hypoglycemia. Informed consent was obtained from all participants and the study protocol was approved by the ethical committee of Aix-Marseille (France). The study was registered under Clinical Trials (ClinicalTrials.gov identifier: NCT04201444).

Protocol

HAIRCUSH was an open-label, multicenter study conducted between March 2021 and May 2022 involving 7 endocrinology departments of French university hospitals. The duration of the study participation for each patient was 3 months. Following enrollment, CushMed patients had 3 monthly outpatient visits with the referral endocrinologist. Patients collected at home 2 24-hour urinary collections for UFC measurement and 2 late-night salivary samples during the 2 days preceding each visit. Saliva and urine samples were returned to the investigator at each visit. Overall, CushMed patients had 6 measurements of UFC, LNSF, and LNSE during the course of the study (days 28, 29, 58, 59, 88, and 89). An 8 AM serum cortisol measurement was also performed in external laboratories before each visit to screen for adrenal insufficiency. It was left to the decision of each investigator to analyze all or part of the saliva and urinary samples to adjust treatment but, in all cases, a duplicate of the samples was stored and frozen. Samples were sent for centralized analysis to Bordeaux University Hospital after completion of the protocol. Hair collection was performed as previously described at the last 3-month visit (14). Following enrollment, CushSurg and CushBla patients carried out only the last 3-month visit with 2 saliva and urine samples collected during the previous 48 hours at home and the hair sample being taken the day of the visit. The 8 AM serum cortisol measurement was not performed in CushSurg and CushBla patients.

Clinical Evaluation

The duration of the period called "control of hypercortisolism" was calculated according to the time elapsed between the start of the study and the date of control of hypercortisolism with pharmacological agents for CushMed patients, the date of surgical remission of hypercortisolism for CushSurg, and the date of BLA in CushBla patients. Daily drug dosage calculations were based on tables of equivalent antihypertensive power for the various antihypertensive drugs that patients were taking (17). The daily defined dose tables were provided by the hypertension unit of Pompidou European Hospital (Paris, France). To evaluate the clinical intensity of Cushing syndrome, we adapted a previously published arbitrary clinical score (18). A score of 1 was recorded in the presence of nonspecific symptoms such as buffalo hump, facial plethora, obesity, glucose intolerance, and hypertension of grade 1 to 2. A score of 2 was recorded in the presence of more specific symptoms such as purple striae, proximal muscle weakness, and fragile skin. A score of 2 was also recorded for diabetes and grade 3 hypertension. The maximum score was 13.

Assays

For saliva assays, a liquefying agent (Sputasol, Thermo Fisher Scientific) was added to 400 µL of saliva samples, which were then incubated for 30 minutes at 37 °C. Then, either saliva or urine samples were prepared for cortisol measurement using automated solid-phase extraction (Oasis HLB, Waters Ltd). Both salivary and urinary extracts were processed by liquid chromatography–tandem mass spectrometry (LC-MS/ MS), with a Prominence Liquid Chromatography system (Shimadzu) and a 5500Qtrap detector (Sciex) for saliva samples and Alliance 2695 and Quattro Micro Waters Systems for urine samples. Cortisol concentration was evaluated using the peak area ratio of the transitions of cortisol and deuterated cortisol from an added internal standard.

Milled hair samples (Precellys 24 instrument) were incubated for 18 hours in 1 mL of methanol. The supernatant was evaporated and the dry residue was resuspended using 1 mL of H_2O/MeOH 98/2 and finally extracted by liquid-liquid extraction with dichloromethane. Assays were performed by LC-MS/MS (Acquity UPLC Class I Plus and XévoTQXS, Waters Systems). HF and HE concentrations were evaluated using the peak area ratio of the transitions of cortisol or cortisone and deuterated cortisol from an added internal standard.

LNSF LC and LNSE MS/MS interassay coefficients of variation (CVs) were less than 10% at 0.9, 2.9, 5.0, and 9.2 nmol/L. The limit of quantification was 0.28 nmol/L. UFC LC-MSMS interassay CVs were less than 10% at 9.5 µg/L and 132 µg/L, respectively. The limit of quantification was 2.5 µg/L. HF and HE interassay CVs were less than 14% at 12.2 pg/mg and 15.8 pg/mg, and the limit of quantification was 1.8 pg/mg and 1.6 pg/mg, respectively.

Statistical Analysis

Continuous variables are expressed as mean ± SD or as median (25th-75th percentiles) for those with skewed distribution. Categorical variables are expressed as the number of participants. Characteristics of participants, clinical score, daily antihypertensive drug dosage, and hormonal investigations were compared according to study groups using the Pearson chi-square test, analysis of variance, or Kruskal-Wallis/Wilcoxon rank sum test. Post hoc comparisons between pairs were made when statistically significant difference was observed across study groups. The Pearson or Spearman method was used to test the correlations between HF and HE.

The upper limit of the normal range of HE concentrations was defined as values at the 97.5th percentile or more among controls from group CushSurg. Then, hormonal assessments, related variability (discussed later), clinical score, and daily antihypertensive drug dosage were compared in patients who were in the higher range vs those in the normal range in CushMed patients. Concentrations of 8 AM serum cortisol, UFC, LNSF, and LNSE were compared within CushMed patients using linear mixed-regression models, taking into account multiple measurements, while adjusting for patient identification and age. The variability of UFC, LNSF, and

Table 2. Variability of urinary free cortisol, late-night salivary cortisol, and late-night salivary cortisone concentrations according to study group

	CushMed	CushSurg	CushBla	*P*
UFC, µg/24 h	7.07 (5.46-11.48)	4.03 (3.06-6.63)[a]	6.31 (1.71-13.9)	.08
LNSF, nmol/L	0.98 (0.65-1.77)	0.16 (0.07-0.56)[a]	0.86 (0.46-1.48)[b]	.003
LNSE, nmol/L	4.46 (3.61-7.89)	1.81 (0.34-3.32)[a]	1.26 (0.21-5.95)[a]	.004

Data presented as median (25th-75th percentiles) and compared using Kruskal-Wallis test.
P less than .05 was statistically significant.
Abbreviations: CD, Cushing disease; CushMed: patients with CD treated with cortisol-lowering drugs, CushSurg: patients in remission of CD following pituitary surgery, CushBla: patients with CD treated by bilateral adrenalectomy; LNSE, late-night salivary cortisone; LNSF, late-night salivary cortisol; UFC, urinary free cortisol.
[a]Post hoc pair-comparisons were performed to detect statistically significant difference vs group 1.
[b]Vs group 2.

LNSE concentrations was expressed for each participant as SD, and compared in different study groups using a linear model. Statistical analyses were performed using JMP software, version 14 (SAS Institute Inc, www.sas.com). Two-sided *P* values less than .05 were statistically significant.

Results

Patients

Patient characteristics are summarized in Table 1. Forty-nine female patients were enrolled in the study: 18 CushMed patients, 15 CushSurg patients, and 16 CushBla patients. Two CushMed patients and one CushSurg patient left the study for noncompliance with the protocol. One CushSurg patient stopped the protocol because of a nonendocrine surgery, one CushBla patient was excluded because she received corticosteroid infiltration in the knee during the study period, and the amount of hair collected in one CushMed patient was insufficient for biochemical analyses.

Finally, data from 44 female patients, aged 49.0 ± 13.2 years, were analyzed (see Table 1). The 16 CushMed patients received cortisol-lowering drugs for 23.5 months (range, 15.0-69.7 months) following pituitary surgical failure (N = 14) or refusal (N = 2). Pharmacological treatments included osilodrostat (2-10 mg/d), metyrapone (500-3000 mg/d), ketoconazole (400-1200 mg/d), association of 750 mg/d of metyrapone and 1200 mg/d of ketoconazole, pasireotide LAR (20 mg/mo), and cabergoline (2 mg/wk) in 6, 4, 3, 1, 1, and 1 patient, respectively. Centralized analysis of urine samples revealed that all UFC measurements were within the normal range of our laboratory (N < 66 µg/24 hours) in 14 of the 16 CushMed patients. One CushMed patient had only a slight increase in 1 of 6 UFCs at the first-month evaluation (1.1× upper limit of normal). Another CushMed patient had increased values in the 2 UFCs performed at the first-month evaluation (1.6× and 2.3× upper limit of normal), but UFCs were normal during subsequent evaluations. The median 8 AM serum cortisol concentration was 377.0 nmol/L (range, 196-636 nmol/L), and no patient had hypocortisolemia dur- ing monthly controls.

Thirteen CushSurg patients were in remission of CD for 42.0 months (range, 13.0-53.0 months) following pituitary surgery, and the 15 CushBla patients underwent BLA 72.0 months (range, 43.0-106.0 months) before the study start. All but one of the CushBla patients underwent unsuccessful pituitary surgery before BLA. The daily dose of hydrocortisone replacement ranged between 15 and 25 mg/d in 14 of 15 patients and only one CushBla patient with the highest BMI (42.3 kg/m²) received 30 mg/d hydrocortisone intake divided into 2 (N = 10) or 3 daily intakes (N = 5), the last intake being a maximum of 5 mg and taking place no later than 5 PM. No patient required increasing hydrocortisone dose during the study period.

Overall Results in the 3 Study Groups

Age and BMI were similar between the 3 study groups (Tables 1 and 2 and Fig. 1). By contrast, the Cushing clinical score was increased in CushMed patients compared to CushSurg and CushBla patients ($P < .0001$). The calculated duration of "control of hypercortisolism" was significantly increased in CushBla patients compared to CushMed and CushSurg patients ($P < .01$), while it was similar between the latter 2 groups.

Median UFC, LNSF and LNSE were significantly different between groups (see Table 1). Post hoc pair-comparison analyses revealed that UFC was higher in CushMed and CushBla patients compared to CushSurg patients. LNSF and LNSE were significantly increased in CushMed patients compared to CushSurg and CushBla patients.

The variability in UFC, LNSF, and LNSE concentrations across samples was increased in CushMed patients compared to CushSurg patients (see Table 2). CushBla patients also had increased variability in LNSF compared to CushSurg patients.

Median HF and HE concentrations were 7.6 ng/mg (range, 3.5-22.9 ng/mg) and 22.1 ng/mg (range, 14.6-49.8 ng/mg) in the whole cohort. HE concentrations were significantly higher than HF concentrations ($P < .0001$) leading to an overall HF/HE ratio of 0.29 (range, 0.20-0.48). HE and HF were correlated in the whole cohort ($r = 0.76$; $P < .0001$) and in each study group ($r = 0.76$; $P < .0001$; $r = 0.81$; $P < .0005$; and $r = 0.56$; $P < .005$ for CushMed, CushSurg, and CushBla, respectively).

Median HF concentration was higher in CushBla patients than the other groups ($P = .002$), while the difference in HF between CushMed and CushSurg patients did not reach statistical significance ($P = .17$). Median HE concentration was higher in CushMed and CushBla patients than in CushSurg patients ($P = .003$).

Subanalysis of Patients Treated With Cortisol-lowering Agents

As only HE concentrations, and not HF concentrations, were significantly different between CushMed and CushSurg patients, we performed a subanalysis comparing CushMed patients based on their HE concentrations. The 97.5th

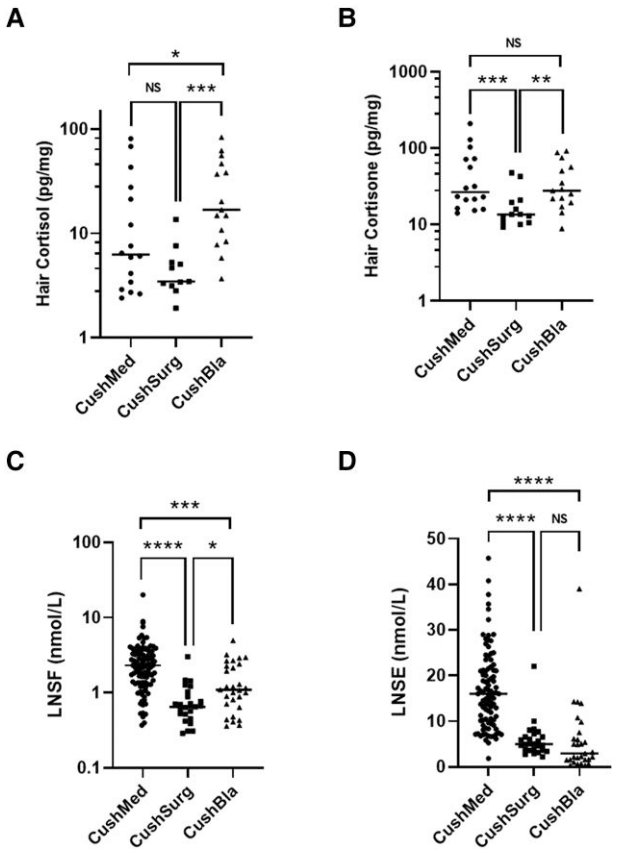

Figure 1. Individual results of endocrine investigations. A, Hair cortisol; B, hair cortisone; C, late-night salivary cortisol (LNSF); D, late-night salivary cortisone (LNSE). CushMed: Patients with Cushing disease (CD) treated with cortisol-lowering drugs; CushSurg: patients in remission of CD following pituitary surgery; CushBla: patients with CD treated by bilateral adrenalectomy. The horizontal line within dots corresponds to the median concentration. Statistical significance: NS, nonsignificant; *P less than .05; **P less than .01; ***P less than .001; ****P less than .0001.

percentile of the HE concentrations observed in CushSurg patients was considered the upper limit of the normal range. Accordingly, we have assumed that patients with HE of 47.8 pg/mg or greater have increased concentrations. Among the 16 CushMed patients, 6 (37.5%) had increased HE concentrations above the normal range. Compared to those with normal HE concentrations, these 6 patients had higher UFC (42.5 [95% CI, 34.0-50.9] vs 28.5 [95% CI, 22.6-34.3] µg/24 h; P = .009) and LNSE concentrations (19.2 [95% CI, 16.3-22.2] vs 15.4 [95% CI, 13.2-17.6] nmol/L; P = .04). By contrast, no significant difference was observed between these 2 subgroups for LNSF concentrations (3.01 [95% CI, 2.18-3.83] vs 2.51 [95% CI, 1.89-3.13] nmol/L; P = .35]) and 8 AM serum cortisol (379 [95% CI, 297-460) vs 360 [95% CI, 300-421] nmol/L; P = .72).

The variability in LNSE concentrations across samples was higher in patients with increased HE compared to those with normal HE (7.6 [95% CI, 5.2-10.1) vs 4.5 [95% CI, 2.6-6.4) nmol/L; P = .04). The variability of UFC and LNSF was almost 2 times greater in patients with higher HE compared to those with normal HE, but the difference did not reach statistical significance (14.7 [95% CI, 6.4-23.1] vs 7.8 [95% CI, 1.3-14.3] µg/24 h; P = .18 and 2.12 [95% CI, 0.67-3.57] vs 1.10 [95% CI, 0.02-2.22]; P = .25; respectively).

From a clinical standpoint, the clinical score was similar between patients with increased and those with normal HE concentrations: 5.5 (2.7-7.5) vs 4.5 (2.7-5.2); P = .38, respectively. Patients with increased HE concentrations had nonsignificant increase in systolic blood pressure (130.0 [120.0-140.0] vs 120.0 [115.8-131.5] mm Hg; P = .32; respectively), and diastolic blood pressure (80.0 [76.7-92.0] vs 75.0 [69.5-84.0] mm Hg; P = .33; respectively). However, compared to patients with normal HE concentrations, patients with increased HE had an increased requirement for antihypertensive drugs (daily drug dosage = 1.0 [0.5-1.4] vs 0.0 [0.0-1.0]; P = .05; respectively).

Subanalysis of Patients Treated With Bilateral Adrenalectomy

HF concentrations in CushBla patients were increased compared to CushMed and CushSurg patients (see Table 1), while HE concentrations were increased only compared to CushSurg patients and were similar to CushMed patients. The median HE/HF ratio was higher in CushBla controls than in CushMed and CushSurg patients (P = .0002).

Despite similar UFC and HE concentrations between CushMed and CushBla patients, CushBla patients exhibited significantly lower LNSF and LNSE concentrations, the latter being similar to that of CushSurg patients. Among CushBla patients, 5 (33.3%) had increased HE concentrations above control concentrations. There was no correlation between the hydrocortisone daily dose (expressed per square meter of body surface) and HE (r = 0.30; P = .28). Similarly, there was no difference in hydrocortisone daily doses between patients with increased HE and those with normal HE concentrations: 13.0 (range, 10.2-13.6) vs 11.7 mg/m²/d (range, 9.9- 14.5), respectively; P = .95.

Discussion

The main findings of our study are that a significant subset of CD patients treated with various cortisol-lowering drugs exhibits persistent mild hypercortisolism despite consistently normalized UFC. Persistent mild hypercortisolism was related to highly variable and frequently increased LNSF and LNSE concentrations, reflecting an altered circadian rhythm of free serum cortisol. This mild hypercortisolism could be diagnosed over a 3-month period by a single HE measurement in a 3-cm scalp hair sample. Increased HE concentrations in patients treated with cortisol-lowering drugs were associated with an increased need for antihypertensive drugs. CushBla patients who received recommended doses of hydrocortisone had increased HE concentrations similar to those of patients treated with cortisol-lowering agents.

Normalization of UFC is the most commonly used biological target in patients treated with cortisol-lowering drugs (1–3, 9). Although 24-hour UFC collection provides an integrated measure of free cortisol production, it ignores variations in cortisol during the day, and small increases in cortisol production, which do not overwhelm the binding capacity of cortisol-binding globulin, may not lead to an increase in UFC. The limited sensitivity of UFC to diagnose mild cortisol excess has been demonstrated in de novo mild CD and early-stage recurrence of CD (10, 11, 19–21). In these conditions, mild hypercortisolism frequently occurs at the time when physiological levels of cortisol reach their nadir and can be diagnosed

with LNSF measurement (10, 11, 19–21). Assessment of salivary cortisone, issued by the rapid oxidization of cortisol by salivary 11β-HSD2, is an improved measure of serum free cortisol (22, 23) and a few studies have suggested that LNSE can diagnose mild night-time hypercortisolism when LNSF and UFC are normal (24–27). Using 6 late-night saliva samples drawn over a 3-month period, we found that pharmacologically treated patients had significantly increased LNSE compared to controls patients surgically cured of CD. These results are consistent with previous studies acknowledging that only 17% to 44% of medically treated patients with normal UFC had normalized LNSF (5, 7, 8). The important variability in LNSF and LNSE concentrations that we observed in these patients is reminiscent of spontaneous fluctuations in cortisol production observed in untreated CD (4, 28) and illustrates that the cortisol-lowering agents do not restore normal pituitary-adrenal axis function (3, 9) but only dampen cortisol production.

Cortisol is incorporated into the hair cells by passive diffusion from the blood, and with hair having a growth rate of roughly 1 cm/month, hair is a suitable matrix to estimate the tissular exposure to cortisol over longer periods of time compared to urine or saliva measurements. HF has been shown to be a sensitive tool to diagnose overt Cushing syndrome with a specific usefulness in patients with intermittent hypercortisolism (12). As in the salivary glands, the 11β-HSD2 enzyme in eccrine sweat glands of the skin generates cortisone from cortisol in hair. Few studies have shown that HE has slightly better diagnostic sensitivity than HF and is able to identify mild hypercortisolism in patients with normal UFC (13, 14).

Overall, patients treated with cortisol-lowering agents had increased HE. The lack of statistical significance of the difference in HF concentrations between CushMed and CushSurg may be due to the small size of our series but could also be interpreted as a higher sensitivity of HE compared to HF, as shown in previous studies in Cushing syndrome (13, 14).

Although almost always within the normal range of our laboratory, UFCs were increased in patients with elevated HE compared to patients with normal HE. Increased and more variable concentrations of LNSF and LNSE were also observed in patients with elevated HE. Thus, similarly to glycosylated hemoglobin, which assesses the global glycemic control over several months in patients with diabetes, a single measurement of HE may identify patients with persistent and fluctuating mild hypercortisolism over periods of several months. Overall, CushMed patients had increased clinical Cushing scores compared to patients surgically cured of CD despite a similar duration of "control of hypercortisolism," suggesting that mild, persistent hypercortisolism may play a role in the maintenance of cortisol-related comorbidities.

The subgroup of CushMed patients with increased HE had, despite a nonsignificant increase in blood pressure, an increased need for antihypertensive drugs compared to patients with normal HE. These results are reminiscent of the finding that CD patients treated with pasireotide LAR and with both normalized UFC and LNSF had greater improvement in blood pressure compared to patients with only normalized UFC (6). We also previously found that, in patients with mild Cushing syndrome of various etiologies and normal or near normal UFC, increased HE was associated with an increased daily drug dosage of antihypertensive drugs (14). From this perspective, it is worth mentioning studies showing that cessation of mild hypercortisolism may induce clinical benefit for CD patients with increased LNSF despite normal UFC (29) and that increased HF and HE in large general populations is associated with cardiovascular risk factors (30, 31).

Also of note, CushBla patients receiving recommended doses of hydrocortisone exhibited increased HF and HE concentrations, a finding suggesting that a 15 to 25 mg daily dosage leads to excessive chronic exposure to glucocorticoids. Two previous studies also found increased HF in adult patients with adrenal insufficiency treated with variable or recommended doses of hydrocortisone (15, 16, 32, 33). Using stable isotope dilution technologies, the mean daily cortisol production rate was estimated to be between 5.7 and 7.0 mg/m² (34). The 15 to 25 mg daily dosage in our patients resulted in a median dose of 12.2/m²/d, which is approximately 1.7 to 2.1 times higher than the physiological production, and that may explain their increased HE. In accordance with this, and contrary to our findings, previous studies conducted on larger cohorts of patients using a wider range of hydrocortisone daily doses found a correlation between HF concentration and hydrocortisone dosage (32, 33). Interestingly, HE concentrations in CushBla patients were similar to that of CushMed patients, but their HF concentrations were greater, this difference being responsible for an increased HF/HE ratio. We speculate that this increased ratio is due to differences in metabolism between exogenously administered hydrocortisone and endogenously secreted cortisol. Indeed, serum cortisol concentrations reach supraphysiological concentrations following the intake of hydrocortisone (35), which may saturate the activity of hair 11β-HSD2 and decrease the transformation of cortisol in cortisone (23). The hepatic first pass of hydrocortisone and exposure to increased activity of the cortisol-regenerative 11β-HDS1 (36) may also participate in the increased HF/HE ratio, as observed for cortisol metabolites in the urine (36) and for the LNSF/LNSE saliva ratio throughout the day (24, 37).

Despite a similar increase in HE concentrations, LNSF and LNSE concentrations in CushBla patients were lower than that of CushMed patients. This finding confirms that, unlike patients receiving cortisol-lowering drugs, the excessive cortisol load occurs within hours of taking hydrocortisone and not during the nighttime period, as expected given the time of taking hydrocortisone. This difference in the circadian timing of cortisol excess may at least partly explain the lower clinical score of CushBla patients compared to that of CushMed patients despite similar increased HE concentrations (38, 39). The longer duration of "control of hypercortisolism" in CushBla patients and sampling variations in the recruitment of patients may also account for this difference.

Our study has several limitations. Its small sample size might have been insufficient to detect differences with a smaller magnitude. Specifically, our normal range of HE concentrations is questionable, so an accurate estimation of the fraction of CushMed patients and with increased HE despite normal UFCs requires large-scale confirmatory studies. The small sample size does not make it possible to compare the effectiveness of the control of hypercortisolism between cortisol-lowering agents. Also, the analysis of the clinical score has the limitation of not considering the clinical score at presenta- tion and the duration of evolution of the hypercortisolism be- fore its control. However, the evaluation of the true active phase of CD, which includes the prediagnosis phase, is always imprecise. Although our clinical score has not been validated, it includes the main elements of Cushing syndrome and has been used in published studies (18).

In conclusion, our study suggests that normalization of UFC should not be the only biochemical target for CD patients treated with cortisol-lowering agents since a statistically significant proportion of them exhibit persistent mild hypercortisolism at least during the nocturnal period. Given the variability in cortisol secretion in these patients, multiple measurements of LNSF and LNSE may be needed to diagnose this persistent, mild hypercortisolism once UFC is normalized. A complementary approach is represented by HE measurements that provide insight into the cumulative tissular exposure to cortisol over months. In CD patients treated with BLA, our data confirm that the hydrocortisone-replacement dose should be lower than that usually prescribed (34).

Future studies are needed to define more precisely the place of HF and HE measurements in the follow-up of CD patients treated with cortisol-lowering agents using a titration procedure or a block-and-replace regimen (3) as well as CD patients treated with BLA and receiving hydrocortisone.

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Disclosures

The authors have nothing to disclose.

Data Availability

The data analyzed during the current study are not publicly available out of consideration of intellectual property, and continuing analyses by the study investigators, but may be available from the last author on reasonable request.

Clinical Trial Information

Clinical trial registration number NCT04201444 (registered December 17, 2019).

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