Hair cortisol and cortisone measurements for the diagnosis of overt and mild Cushing's syndrome

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Abstract

Objective: Hair cortisol (HF) and cortisone (HE) measurements reflect tissular exposure to cortisol over months and are increased in overt Cushing's syndrome (CS). No data is available in mild CS. We compared the diagnostic performance of HF and HE between patients with overt or mild CS.

Design: Single centre retrospective study.

Methods: HF&HE were measured by LC-MS/MS in 48 consecutive adult females with Cushing's disease (CD), ectopic ACTH syndrome, secreting adenomas and carcinomas, and adrenal incidentalomas. All had impaired dexamethasone suppression tests. Overt CS ($n = 25$) was diagnosed in front of specific symptoms, a mean UFC (>1.5 ULN) and increased midnight serum cortisol or salivary cortisol. Mild CS ($n = 23$) was diagnosed in patients lacking specific symptoms and displaying at least one additional biological abnormality including mildly increased UFC (≤ 1.5 ULN), increased midnight serum cortisol or salivary cortisol and suppressed plasma ACTH in patients with adrenal tumours. In this study, 84 healthy subjects and obese patients served as controls.

Results: HF and HE showed roughly similar performance in overt CS (92 and 100% sensitivity, 91 and 99% specificity, respectively). HF and HE were lower in mild CS but higher than in controls ($P < 0.01$). HE was correlated with midnight serum cortisol ($P < 0.02$) and volume of adrenal incidentalomas ($P < 0.04$) but not with UFC. HF and HE had 59% and 68% sensitivity, and 79 and 94% specificity, respectively, for the diagnosis of mild CS. Contrary to UFC, both HF and HE were in the range of overt CS in 11/23 patients with mild CS. Patients with mild CS and increased HE required more antihypertensive treatments and showed worser lipid profiles than patients with normal HE.

Conclusions: HF and HE measurement performed better in overt than in mild CS but is a useful adjunct to diagnose mild CS and to identify adrenocortical incidentalomas responsible for excessive cortisol exposure.

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Introduction

Numerous biochemical investigations are available for diagnosis of Cushing's syndrome (CS), including UFC (urinary free cortisol) measurements, serum cortisol concentration after DST (dexamethasone suppression

test), late-night salivary cortisol (LNSC) or midnight serum cortisol (MSC) assays (1). One major limitation of these tools is that they only measure point cortisol concentrations or short-term cortisol exposure.

The inherent variability in cortisol secretion during CS is a major issue that justifies the recommendations to perform at least 2 UFC or LNSC tests for its diagnosis (2, 3, 4). To circumvent this drawback, measurement of cortisol in hair has been proposed. Cortisol is incorporated into the growing hair cells by passive diffusion from blood capillaries, and with the hair having a growth rate of approximately 1 cm/month, hair is a suitable matrix for the estimation of mid- to long-term tissular exposure to circulating cortisol. Three studies have reported on the diagnostic accuracy of cortisol measurement in a single hair sample (5, 6, 7) and its usefulness in patients with intermittent/cyclical CS (5, 6, 8).

The 11 β hydroxysteroid dehydrogenase type 2 enzyme (11 β -HSD type 2) is expressed in eccrine sweat glands of the skin, resulting in local generation of cortisone from cortisol in hair (9). Whether measurement of hair cortisone (HE) may be more useful than measurement of hair cortisol (HF) has been addressed recently in a single study which suggested that the diagnostic performance of HE measurement is superior to that of HF (7).

Mild CS (also incorrectly termed 'subclinical CS') is characterised by the lack of specific features of CS and a mild hypercortisolism resulting in diagnostic complexity (10, 11). Mild CS is associated with various pathologic situations, the most frequent being adrenal incidentalomas (10, 12). These tumours may exhibit impaired cortisol suppression following the 1 mg DST, a finding consistent with cortisol secretory autonomy (12) but that frequently contrasts with normal UFC and LNSC levels (10, 11, 12, 13, 14), a finding that raises the question of whether some of these tumours induce definitive hypercortisolism (12, 13, 15, 16, 17). Early-stage recurrence of Cushing's disease (CD) is another difficult diagnostic situation characterised by mild and highly fluctuating cortisol excess. In addition, it has been shown increased UFC is often a late event during recurrence of CD (18, 19). Last, true *de novo* mild CD may be responsible for a minimal excess of cortisol production resulting in normal UFC (11, 20, 21). To date, no data concerning HF and HE measurement in mild CS is available. We hypothesised that the ability of HF and HE measurement to assess the long-term exposure to cortisol may help to diagnose cortisol excess in this situation and compared the diagnostic performance of HF and HE by liquid chromatography coupled with mass spectrometry (LC-MS/MS) between patients with overt CS and mild CS.

Methods

Participants

Consecutive patients who were referred to the Endocrinology Department of the University hospital of Bordeaux for suspicion of CS and investigation of adrenal incidentalomas were asked to give a scalp-hair sample for glucocorticoids analysis. All patients gave informed consent before participation in the study and biochemical data were collected under conditions of regular clinical care, with local ethics committee of Bordeaux approval for the use of those data. Medical records were reviewed retrospectively. Only females were included in the study and patients who used topical or systemic corticosteroids were excluded.

Forty-eight female patients were included and classified as having overt CS or mild CS. All had a serum cortisol >50 nmol/L after the 1 mg DST. Overt CS was diagnosed in the presence of specific symptoms of cortisol excess after examination by an experienced endocrinologist and various abnormal biochemical tests, including a mean of 2–5 UFC >1.5 the upper limit of normal range (ULN) and increased MSC (≥ 180 nmol/L) or increased LNSC above our reference range. Mild CS was diagnosed in the absence of specific clinical symptoms and at least one of the following endocrine alterations: mildly increased UFC (mean of 2–5 UFC ≤ 1.5 ULN), plasma ACTH concentration ≤ 2.2 pmol/L in patients with adrenocortical tumours, increased MSC or increased LNSC.

The control population consisted of 84 females: 26 obese and/or diabetic patients, 4 patients referred for suspicion of CS and 54 healthy controls. None of these patients suffered from alcoholism, depression or were receiving topical or systemic corticosteroids. All patients had cortisol concentrations <50 nmol/L following the 1mg DST or had normal LNSC.

Hair collection

In all participants, a lock of hair as thick as a pencil and measuring approximately 3 cm in length was cut from the posterior vertex as close to the scalp as possible. Hair samples were taped on a sheet of paper and the proximal side of the hairs was indicated. The hair samples were stored in a dry envelope at room temperature until analysis.

Assays

Serum cortisol was assayed using an automated analyser (Architect, Abbott). ACTH serum concentrations were

assayed by IRMA (ACTH RIA, BRAHMS, Germany) according to the manufacturer's instructions.

For saliva assays, a liquefying agent (Sputasol, Thermo Fisher Scientific) was added to 400 µL of saliva samples which were then incubated 30 mn at 37°C. Then, either saliva or urine samples were prepared for cortisol measurement using automated solid-phase extraction (Oasis HLB, Waters Ltd, Elstree, Hertfordshire, UK). Both salivary and urinary extracts were processed by LC–MS/MS, with a Prominence Liquid Chromatography system (Shimadzu, Nakagyo, Japan) and a 5500Qtrap detector (Sciex, DC, USA) 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, Montigny-le-Bretonneux, France) were incubated for 2 h in a dithiothreitol solution (0.77 mg/mL NaOH 0.1N). The supernatant was extracted by liquid–liquid extraction with dichloromethane. Assays were performed by LC–MS/MS (Prominence Liquid Chromatography system, Shimadzu and 5500 Qtrap detector, Sciex). Cortisol and cortisone concentrations were evaluated using the peak area ratio of the transitions of cortisol or cortisone and deuterated cortisol from an added internal standard.

Serum cortisol assay Interassay coefficients of variation (CV) were <5% at 97, 394 and 800 nmol/L. The limit of quantification is 11 nmol/L. Plasma ACTH assay Interassay CVs are <5% at 7.7 and 73 pmol/L. The limit of quantification is 1.16 pmol/L. LNSC LC-MSMS assay interassay CVs were <10% at 0.9, 2.9 and 9.2 nmol/L. The limits of quantification is 0.28. UFC LC-MSMS assays interassay CVs were <10% at 9.5 and 132 µg/L, respectively. The limits of quantification was 2.5 µg/L. HF

and HE assays' interassay CVs were <20% at 22 and 40 pg/mg and the limits of quantification was 6.0 and 2.0 pg/mg, respectively.

Statistical analysis

ACTH and cortisol concentrations below the detection limit of the assay were set to the lowest detection value for calculation purposes. The volume of adrenal nodules was estimated from dedicated CT scans using the formula: $4/3 \times \Pi \times r^3$. For the purpose of calculation, the sum of volumes was used in those patients with multinodular disease. Daily drug dosage (DDD) calculations were based on tables of equivalent antihypertensive power for the various antihypertensive drugs that patients were taking (22). The daily defined dose (DDD) tables were provided by the hypertension unit of Pompidou European Hospital (Paris, France). Statistical analyses were performed using GraphPad Prism version 8.4.2 (GraphPad Software) and MedCalc version 19.4.1 (MedCalc Software Ltd, Ostend, Belgium). All quantitative variables are expressed as median and interquartile range (IQR). Chi-square, Spearman correlation, Passing–Bablok regression, Mann–Whitney, Kruskal–Wallis, ANOVA test after Log-transformation of data when necessary followed by *post hoc* tests were used as appropriate. Cut-off values used for the calculation of sensitivities and specificities were determined by receiver operating characteristic (ROC) curves. All outcomes were considered statistically significant where *P* values were <0.05.

Results

The characteristics of control subjects and patients are summarised in Table 1 and Fig. 1.

Table 1 Characteristics of control subjects and patients. Data are shown as median (IQR).

	Controls	Overt CS	Mild CS	<i>P</i> values
<i>n</i>	84	25	23	
Age, y	43.4 (28.2–56.5) ^a	53.0 (35.5–58.5)	55.0 (44.0–68.0)	<0.02
BMI, kg/m ²	24.0 (21.0–30.7) ^b	29.8 (26.6–33.3)	29.4 (26.2–34.3)	<0.01
DST, nmol/L	27 (19–28) ^c	367 (302–526) ^d	165 (94–269)	<0.01
UFC, ULN		3.9 (2.5–7.1)	0.5 (0.2–0.8)	<0.01
MSC, nmol/L		475.0 (392.5–565.0)	194.3 (146.5–293.5)	<0.01
HF, pg/mg	8.7 (6.7–11.8) ^e	58.8 (25.3–78.0) ^f	14.5 (6.2–29.7)	<0.01
HE, pg/mg	13.9 (11.0–16.6) ^g	68.2 (39.0–117.6) ^h	27.8 (12.3–41.7)	<0.01
Ratio HF/HE	0.7 (0.5–0.9)	0.6 (0.5–0.9)	0.6 (0.3–0.9)	0.6

**P* < 0.02 vs mild CS; ^b*P* < 0.01 vs overt CS; ^c*P* < 0.01 vs overt CS and mild CS; ^d*P* < 0.01 vs mild CS; ^e*P* < 0.03 vs mild CS; ^f*P* < 0.01 vs controls and mild CS;

^g*P* < 0.01 vs overt CS and mild CS; ^h*P* < 0.01 vs mild CS.

CS, Cushing's syndrome; DST, serum cortisol concentration following the 1 mg overnight dexamethasone test; HE, hair cortisone; HF, hair cortisol; MSC, midnight serum cortisol; UFC, 24-h urinary free cortisol; ULN, upper limit of normal.

Figure 1

Distribution of hair cortisol (A) and hair cortisone (B) according to (A) urinary free cortisol (UFC) and midnight serum cortisol (MSC) (B); mild Cushing's syndrome: left panels, overt Cushing's syndrome: right panels. Coefficients of correlation are presented on each plot.

Control population

HE concentrations were higher than HF concentrations ($P < 0.01$). We found no difference in HF and HE concentrations between obese and lean/overweight subjects: HF=10.0 pg/mg (5.9–19.7) vs 8.6 pg/mg (7.0–11.3), respectively; $P = 0.4$; HE=15.1 pg/mg (7.7–19.3) vs 13.9 pg/mg (11.1–16.2), respectively; $P = 0.7$ nor between older (age ≥ 60 years) and younger (age < 60 years) controls: HF=8.8 pg/mg (5.9–11.3) vs 8.9 pg/mg (6.9–12.6), respectively; $P = 0.6$; HE=13.9 pg/mg (8.7–18.8) vs 13.9 pg/mg (11.0–16.4), respectively; $P = 0.8$). In addition, no correlation was found between HF and HE levels and BMI nor age (HF: $r = -0.03$, $P = 0.8$ and $r = -0.15$, $P = 0.2$; respectively; HE: $r = 0.02$, and $r = 0.01$, $P = 0.9$ for both; respectively). Consequently, the values of HF and HE

Figure 2

ROC curves for overt (A) and mild (B) Cushing's syndrome. HE, hair cortisone; HF, hair cortisol. The values quoted in the figures correspond to the specific HE and HF diagnostic thresholds with best compromise between sensitivity and specificity.

from the different control groups were pooled as a single cohort.

Overt Cushing's syndrome

According to our criteria, 25 patients had overt CS: 17 cases of *de novo* CD and 1 recurrence after surgical remission, 2 with ectopic ACTH syndrome, 5 with cortisol-secreting tumours (2 adenomas, 3 carcinomas). CD was confirmed by histopathological analysis or post-operative corticotrophic insufficiency in 15 cases, and the results of bilateral inferior petrosal sinus sampling in 2 cases. Patients with overt CS had higher BMI than controls but were of similar age (Table 1).

HE concentrations were higher than HF concentrations ($P < 0.02$). HF and HE were associated ($r = 0.74$, $P < 0.01$). HF was also correlated with UFC ($r = 0.46$, $P < 0.03$) and MSC ($r = 0.55$, $P < 0.01$), as opposed to HE ($r = 0.26$, $P = 0.2$ and $r = 0.34$, $P = 0.11$; respectively) (Fig. 1). There was no statistically significant linear association between the HF/HE ratio and UFC ($r = 0.39$, $P = 0.09$). However, the half of the patients with the highest HF values had higher HF/HE ratio than the half of the patients with the lowest HF values=0.81 (0.58–1.71) vs 0.54 (0.44–0.64), respectively; $P < 0.01$.

HF and HE concentrations were higher in overt CS than in controls ($P < 0.01$) while HF/HE ratio were similar (Table 1). There was no significant difference between the area under the curve (AUC) of ROC curves for HF and HE (0.96 (95% CI : 0.90–1.00) vs 0.99 (0.99–1.00), respectively; $P = 0.11$) (Fig. 2A). With an optimal cut-off of 18.1 pg/mg, HF measurement displayed a sensitivity of

Figure 3

Hair cortisol and cortisone concentrations (logarithmic scale) in control subjects, patients with overt and mild Cushing's syndrome (CS). Values of patients with Cushing's disease are shown as closed circles, those of patients with adrenal disease are shown as open circles and those of patients with ectopic ACTH syndrome are shown as closed squares. The dotted horizontal lines represent the diagnostic thresholds issued from ROC curves analysis in overt CS (large dashes) and mild CS (small dashes).

92.0% and a specificity of 91.3%. With an optimal cut-off of 28.2 pg/mg, HE displayed a sensitivity of 100.0%, and a specificity of 98.8% (Fig. 3). Two of the 25 patients had normal HF concentrations (7.5 and 13.8) associated with increased HE concentrations (29.5 and 34.8, respectively). As expected, patients with ectopic ACTH syndrome and intense hypercortisolism had the highest HF concentrations (Fig. 3).

The sensitivity of UFC to diagnose overt CS (e.g. mean UFC >1.5 ULN), based on 49 analyses performed in the 25 patients during the same period, was 93.9%. Three patients had intermittent hypercortisolism with one UFC <1.5 ULN. HE and HF measurements covering this period were increased in these three patients.

Mild Cushing's syndrome

Twenty-three patients had mild CS: 8 patients had CD (5 *de novo* disease and 3 early-stage recurrence after surgical remission) and 15 had adrenal disease: 8 patients with unilateral adrenocortical incidentalomas, 6 with bilateral macronodular adrenal hyperplasia (BMAH) and 1 with primary pigmented nodular adrenocortical disease. Definitive diagnosis of CD or its recurrence was based on histopathological analysis and/or corticotrophic insufficiency after re-operation.

HE concentrations were higher than HF concentrations ($P < 0.01$). HE was positively correlated with HF ($r = 0.78$, $P < 0.01$) and MSC ($r = 0.51$, $P < 0.02$) but not with UFC ($r = 0.34$, $P = 0.12$). HF was not associated with MSC nor UFC ($r = 0.39$, $P = 0.09$ and $r = 0.12$, $P = 0.60$, respectively) (Fig. 1).

HF and HE concentrations were higher in patients with mild CS compared to controls but lower than in patients with overt CS (Table 1). HF/HE ratio were similar between these groups (Table 1). Following ROC analysis of data from controls and mild CS patients the AUC of HF and HE did not differ significantly (HF 0.64 (0.48–0.80) vs HE 0.75 (0.59–0.90), $P = 0.3$) (Fig. 2). With an optimal cut-off of 12.3 pg/mg, HF measurement displayed a sensitivity of 59.1% and a specificity of 78.8%. With an optimal cut-off of 21.7 pg/mg, HE displayed sensitivity of 68.2% and a specificity of 94.0% (Fig. 3). Three patients had normal HF concentrations (6.3, 8.9 and 9.6 pg/mg) in the presence of increased HE concentrations (22.1, 23.0, 29.1 pg/mg, respectively). Conversely, one patient had a normal HE concentration at 9.9 pg/mg while HF was increased at 28.3 pg/mg. During the period covered by hair analysis, the sensitivity of UFC based on 40 samples was 27.5%

Comparison of biological investigations between patients with mild Cushing's disease and cortisol secretory autonomy

Compared to patients with adrenal disease, patients with CD had similar cortisol levels following the 1 mg DST (155.0 (94.0–260.0) vs 170.5 nmol/L (97.8–308.8), respectively; $P = 0.6$), increased UFC (0.31 (0.19–0.56) vs 0.88 ULN (0.54–1.33), respectively; $P < 0.01$) and increased MSC (178.0 (129.5–231.0) vs 284.3 nmol/L (182.–436.8), respectively; $P < 0.02$). Cortisol levels after DST were >140 nmol/L in 10/15 patients and between 70 and 140 nmol/L in the 5/15 patients with adrenal disease. Fourteen of these 15 patients had 08:00 h plasma ACTH <2.2 pmol/L.

The concentrations of HF and HE were not significantly different between patients with CD and adrenal disease: HF 21.2 (10.1–58.2) vs 11.0 pg/mg (5.3–21.3), respectively; $P = 0.15$ and HE 33.6 (22.3–60.7) vs 24.8 pg/mg (9.9–40.1), respectively; $P = 0.24$. Seven of eight patients with CD and 8/15 patients with adrenal disease had increased HE. Interestingly, 5/8 patients with CD and 6/15 patients with adrenal disease had increased values of HE that were in the range of patients with overt CS. During the period covered by hair analysis, the sensitivity of UFC 37.5% in patients with CD and 12.5% in patients with adrenal tumours.

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Table 2 Metabolic status of patients with mild CS and normal (<28.2) or increased (≥28.2) concentrations of Hair Cortisone (HE).

	HE (pg/mg)		P-values
	< 28.2 (n = 11)	≥ 28.2 (n = 8)	
BMI, kg/m ²	26.2 (20.9–33.6)	30.8 (29.0–35.3)	0.1
Hypertension, n (%)	8 (72.7)	7 (87.5)	
Antihypertensive medication, DDD	1.0 (0.0–2.0)	3.0 (1.8–4.0)	0.05
SBP, mmHg	141.0 (116.0–145.0)	131.5 (125.5–146.5)	0.8
DBP, mmHg	73.0 (67.0–90.0)	79.5 (68.5–88.3)	0.8
Diabetes mellitus, n (%)	1 (9.1)	0 (0.0)	
FBG, g/L	0.86 (0.79–1.01)	0.94 (0.87–1.04)	0.4
HbA1c, %	5.9 (5.2–6.3)	5.8 (5.6–6.3)	0.98
Triglycerides, g/L	1.01 (0.61–1.52)	2.11 (1.28–2.42)	< 0.01
LDL cholesterol, g/L	1.23 (1.07–1.53)	1.35 (1.18–1.77)	0.4
HDL cholesterol, g/L	0.57 (0.53–0.72)	0.47 (0.41–0.62)	P = 0.06

Quantitative data are shown as median (IQR).

DDD, daily defined dose of antihypertensive treatment; DSP, diastolic blood pressure; FBG, fasting blood glucose; SBP, systolic blood pressure.

The estimated volume of adrenal nodules was 12.2 (8.2–27.1) vs 55.3 cm³ (16.9–162.5) in patients with unilateral vs bilateral disease, respectively ($P < 0.02$). There was no significant association between the estimated volume and serum cortisol concentrations following the 1 mg DST ($r = 0.12$; $P = 0.67$). Cortisol concentrations following the DST were similar between patients with unilateral and bilateral adrenal lesions (174.5 nmol/L (83.6–268.5) vs 150.5 nmol/L (94.5–258.6), respectively; $P > 0.99$). HF and HE were associated with the volume of nodules: $r = 0.63$ and 0.59 , respectively; $P < 0.04$ for both. HE but not HF, was increased in bilateral adrenal disease compared to unilateral disease: HE = 30.3 (23.2–46.6) vs 11.3 pg/mg (6.5–21.3), respectively; $P < 0.05$ and HF = 16.1 (6.6–34.0) vs 6.1 pg/mg (4.2–13.5), respectively; $P = 0.18$.

Clinical features in patients with mild CS and increased HE

We compared several cortisol-induced cardiovascular risk factors between patients with mild CS and increased or normal HE (Table 2). To avoid the confounding effects of sequelae arising from a previous history of CD, only patients with *de novo* MCD and with adrenal disease were considered. Despite similar blood pressure values, patients with increased HE had an increased daily drug dosage of antihypertensive drugs, increased plasma triglyceride concentrations and a trend towards lower HDL concentrations. The median DDD of antihypertensive drugs decreased from 1.8 to 0.6 in 12 of these patients who underwent surgery (pituitary surgery in 5, resection of unilateral adenoma in 5 and unilateral adrenalectomy for BMAH in 2 patients).

Discussion

Overall, our study confirms the excellent performance of hair cortisol analysis for the diagnosis of overt CS and provides complementary insights into the respective diagnostic value of HF and HE measured by LC–MS/MS. We found that the performance of HF&HE measurement was lower in mild CS than in overt CS. However, it was clearly better than that of UFC for the diagnosis of mild *de novo* CD and CD in early-stage recurrence. Our results further demonstrate the heterogeneity in cortisol secretion within adrenal incidentalomas associated with cortisol secretory autonomy (CSA). In this situation, HF&HE identified patients with excessive tissular exposure to cortisol and, possibly, increased cortisol-related comorbidities.

Measurement of HF and HE was associated with sensitivities of 92 and 100% and specificities of 91 and 99% for diagnosis of overt CS. The number of patients in our study was limited but this performance compares well with the 81–93% sensitivity and 88–98% specificity reported in the three published studies measuring hair cortisol using different techniques (immunoassay or LC-MS/MS) in 14–70 patients with overt CS (5, 6, 7). Interestingly, the sensitivity of a single HF&HE measurement in our cohort was roughly comparable to that of several UFC determinations performed in the same period. Cortisone in scalp hair results either from diffusion of serum cortisone or conversion of cortisol by local 11β-HSD2 (9). As described for salivary cortisol and cortisone (1, 23), the typically higher HE concentrations than HF reflects the activity of 11β-HSD type 2 in the scalp that amplify the cortisol signal. One recently published study found a slightly better performance of HE measurement over HF, using LC-MS/MS, when the results

of two independent cohorts of 19 and 70 patients with CS were pooled (7). We also found a slightly better diagnostic accuracy for HE measurement over HF, though this did not reach statistical significance, a finding that may have been due to the limited size of our patient cohort. Whether HE provides a substantial diagnostic improvement over HF measurement is therefore a matter for future research, as well as comparison of measurements of HF using immunoassays with HF and HE measurement using LC-MS/MS. Interestingly, we found that patients with the highest HF concentrations displayed higher HF/HE ratio as compared to patients with lowest HF values. This may reflect the limited ability of the scalp 11 β -HSD type 2 to convert HF to HE at relatively high HF levels as described for the kidney in patients with Cushing's syndrome (24).

Hair sampling has a number of advantages: it is non-invasive and does not require specific timing, hair samples can be stored at room temperature and be sent by mail. Above all, HF and HE measurements reflect long-term glucocorticoid exposure and are not influenced by acute stressors. As illustrated in a few patients in our series, this unique feature could make it possible to substitute a single hair sample for several urine or saliva samples that are necessary in those patients with variable hormonogenesis (3, 2, 5, 6, 8, 11). However, HF and HE measurement suffers from several limitations such as the cumbersome and time-consuming preparation and extraction of hair samples. This particular matrix sample and multiple steps of the assay (sample preparation, sample weighing, standard preparation, extraction, chromatography, detection by LCMS) often leads to higher interassay variability with coefficients of variations ranging between 15 and 20% (25, 26). It has also been shown that frequent hair washing (27) and dye hair may lead to hair cortisol washout. Last, cortisol incorporation in hair shaft, hair growth rate and matrix composition depend on the ethnic group and age (28). All these issues contribute to intra and inter-individual variability resulting in decreased diagnostic performance. In our opinion and in the context of clinical suspicion of CS, HF and HE measurements should therefore be reserved for use in difficult diagnostic cases such as cyclic Cushing's syndrome and performed in expert laboratories taking into account the factors of variability.

Mild CS represents a complex diagnostic situation that requires the use of specific biochemical investigations, and eventually the use of different thresholds than those used for diagnosis of overt CS (10, 11, 12, 14, 20). Indeed, the diagnosis of CD of mild intensity requires repeated measurements of UFC (which may be impractical), and

LNSC in addition to DST (11). An increase in UFC is also a late event during post-operative recurrence of CD and diagnosing early-stage recurrence, a phase that is characterised by intermittent cortisol overproduction, also requires multiples LNSC measurements with specific thresholds to be captured (1, 2, 11, 18, 19, 30). Although conducted in a small series, our results suggest that HF and HE may be of diagnostic utility since of the 7/8 patients who displayed increased values, 5 of these were in the range of overt CD.

Depending on the series and criteria used, mild CS has been described in 5 to 50% of patients with adrenal incidentalomas (10, 12). The ESE has proposed a definition of this condition as ACS, a terminology that reflects the pathophysiology of the disease, for example, cortisol secretion disconnected from control by the HPA axis, as reflected by impaired cortisol suppression following the 1mg DST (12). However, whether ACS results in definitive hypercortisolism is debatable since UFC and LNSC are normal in the majority of patients (10, 13, 31). These discrepancies may result from a variety of causes including false positives of the DST (4, 12, 13, 32). Additionally, one common limitation of LNSC and UFC measurements is that they evaluate point cortisol production in fluids where cortisol circulates or is excreted and may ignore intermittent cortisol overproduction resulting in tissular accumulation. Using the unique characteristic of the hair matrix to assess the long-term tissular exposure to cortisol, we found that roughly half of the patients with ACS had increased HF&HE that were in the range of patients with overt CD, in contrast to normal or borderline UFC. Consistent with this, HE was associated with the volume of adrenal lesions, an estimation of the secretory mass, and was greater in bilateral compared to unilateral incidentalomas. A study using six daily saliva samples collected from awakening to night, showed that patients with ACS had increased AUC for salivary cortisol due to increased morning levels, while LNSC was identical to that of non-functioning incidentalomas (33). Aside from unrecognised intermittent cortisol over-production, this secretory profile may also explain increased HF and HE over a period of months contrasting with normal LNSC and normal UFC due to the limited spillover of cortisol into urine. Measurement of HF and HE may therefore be substituted for multiple repeated biochemical investigations to characterise the secretory status in ACS (12). Similarly to our findings in overt CS, the slightly better performance of HE measurement as compared to HF for the diagnosis of mild CS did not reach statistical

significance, a result that could be due to the limited size of our cohort of patients and needs to be addressed in larger independent studies.

Increased long-term cortisol exposure within normal range levels, evaluated by HE in large populations, is associated with cardiovascular risk factors (34). Interestingly, increased HE in our patients with mild CS was associated with potent cortisol-induced comorbidities such as the intensity of hypertension and dyslipidemia. Furthermore, a reduction in the DDD of antihypertensive drugs was observed after cure of mild CS. These findings are unsurprising in view of the frequency of hypertension in CD and ACS, the frequent improvement of hypertension after excision of adrenal incidentalomas with ACS (12, 35) and the clinical benefit of treatment of mild CD (19). Assessing whether increased HF and HE might serve as a predictor of clinical improvement following surgery of adrenal incidentalomas with ACS could be an important aim of future studies, given the poor predictive value of the usual biochemical investigations (13, 35, 36).

Our study does, however, suffer from various limitations including those inherent to a retrospective study and the limited size of our cohort of mild CS patients. Our results should therefore be considered preliminary and require confirmatory studies with larger cohorts. Only female patients were included in our study and our results cannot be extrapolated to male patients. Gender-specific thresholds should be determined since HF concentrations are increased in males compared to females (28, 37, 38). Our study included mainly healthy subjects and obese patients without disturbance of the HPA axis function as the control group. In the context of ACS, complementary studies of patients with non-secreting adrenal incidentalomas will be necessary. Although consecutive patients with overt CS have been studied, a referral bias may have positively influenced the diagnostic accuracy of HF and HE compared to a more general real-life practice that would be enriched with patients with an unconfirmed suspicion of CS (39). Additionally, chronic stress and depression may increase HF levels (38). Establishing thresholds after the inclusion of patients with these pathologies will be important to enable the differential diagnosis between mild CD and 'pseudo-Cushing's syndrome' (29, 40). Contrary to our findings, some (though not all) authors have reported a positive correlation between HF levels and age or massive obesity (41, 42). Therefore, establishing appropriate normative ranges of concentrations in various populations, as has been published for LNSC and salivary cortisone, will be important to assess the diagnostic value

of HF and HE measurements in mild CS (43). Last, the relatively important inter-assay variability of HF and HE measurements discussed previously may represent a limitation for an accurate measurement of mildest tissular overexposure to cortisol.

In conclusion, our study confirms the excellent performance of HF&HE assay for the diagnosis of overt CD. Given its convenience and high sensitivity, we believe that the overnight DST remains the first-line test of choice for the screening of overt CS, mild CS and ACS (1, 4, 11, 12). However, due to limitations in specificity, complementary investigations are needed to ensure definitive diagnosis (1, 4, 12, 13). HF and HE may also be definitively increased in patients with mild CD and may therefore be a useful adjunct for this diagnosis and a substitute for repeated biochemical evaluations, including repeated LNSC measurement (11, 18, 21). Amongst patients with ACS in whom LNSC is often noncontributive (14, 31, 33), HF and HE measurement is able to identify individuals with tissular over-exposure to cortisol. Whether increased HF&HE in these patients is an indicator of clinical severity, favouring adrenal surgery, remains to be studied. Recent steroid profiling analysis has also suggested that measurement of other glucosteroids (such as corticosterone) may identify patients with ACS at risk of their cardiovascular status worsening with time (44). Measurement of the cumulative deposition of these steroids in scalp hair may also be an area for future research.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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