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An accurate, non-invasive approach to diagnose Cushing's syndrome in at-risk populations

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ABSTRACT

Background: The prevalence of Cushing's syndrome (CS) in at-risk populations in developing countries remains uncertain. Evening urinary cortisol ($UFC_{22–23}$) and salivary cortisol after treatment with 1-mg DST (SAF_{dex}) have seldom been used as diagnostic tools in these populations.
Objectives: (1) To establish the prevalence of CS in adults with cortisol-related morbidities using $UFC_{22–23}$ and SAF_{dex} as markers along with all first-line diagnostic tests recommended for CS; and (2) to assess the performance of each test and define a non-invasive diagnostic approach for CS in at-risk outpatient subjects.
Methods: A total of 128 outpatients were evaluated, including type 1 and 2 diabetic patients with poor metabolic control (DM_1 and DM_2), hypertensive subjects with central obesity (HBP) and premenopausal women with osteoporosis (OS). Controls included 100 healthy volunteers and 23 patients with CS. Total urinary cortisol (UFC), $UFC_{22–23}$, late-night salivary cortisol (SAF_{23}) and suppression of cortisol levels in saliva (SAF_{dex}) and serum (F_{dex}) after treatment with 1-mg DST were assessed.
Results: CS was diagnosed in one DM_2 and one HBP patient; both women exhibited central obesity. Among CS patients, UFC showed more within-person variability than $UFC_{22–23}$ or SAF_{23}. $UFC_{22–23}$ and SAF_{23} were positively and significantly correlated in all groups ($r \geqslant 0.70$; $p \leqslant 0.0001$). $UFC_{22–23} > 44.0$ ng/mg creatinine or $SAF_{23} > 3.8$ nM were 100% sensitive (S) and specific (E) for CS. Furthermore, $SAF_{dex} > 2.0$ nM or $F_{dex} > 50.0$ nM were 100% S and 97.3% E for CS.
Conclusion: CS was diagnosed in 1.5% of at-risk patients. The combination of $UFC_{22–23}$ or SAF_{23} with SAF_{dex} offers a non-invasive diagnostic tool to assess cortisol nadir and feed-back status in outpatients.

1. Introduction

Over the last decade, an unexpectedly high incidence of Cushing's syndrome (CS) has been found in certain high-risk populations, namely, patients with symptoms related to cortisol excess, including poorly controlled diabetes mellitus, resistant hypertension and osteoporosis [1–9]. International guidelines recommended testing for CS in patients with unusual features for their age (e.g., osteoporosis, hypertension, type 2 diabetes mellitus or kidney stones) [10]. However, to our knowledge, this practice has not been fully extended to developing countries.

Cortisol secretion is episodic, with a notable circadian rhythm, and responds to stress. Maximal serum cortisol concentrations are present from 05.00 to 10.00 h; secretion declines thereafter, such that the lowest levels occur from 22.00 to 04.00 h [11–13]. The absence of this circadian rhythm is an important feature observed in CS patients with various disease aetiologies. The status of the cortisol circadian rhythm can be assessed in a non-invasive manner through the measurement of late-night salivary cortisol (SAF_{23}) and 22.00–23.00 h urinary cortisol ($UFC_{22–23}$) levels. SAF_{23} reflects the free fraction of circulating cortisol, whereas $UFC_{22–23}$ is an integrated measurement of the biologically active steroid filtered into the urine. Both measurements have been useful in screening outpatients for CS [9,14–17], but their performances have not been evaluated simultaneously, limiting their use in patients with kidney or salivary gland disorders. The physiological negative feedback of cortisol on the hypothalamic–pituitary-adrenal axis is routinely assessed in serum samples (F_{dex}) after overnight treatment with 1-mg oral dexamethasone (1-mg DST). Levels of salivary cortisol after treatment with 1-mg DST (SAF_{dex}) have proved to be as sensitive and specific as F_{dex} for excluding CS [17,18]. However, this test has seldom been applied to at-risk patients [19].

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The interpretation of biochemical diagnostic tests relies on the rigorous standardisation of sampling protocols and assay methodology. Sampling through the receiver operating characteristics curve (ROC) provides the optimal threshold for defining a test's ability to properly diagnose the true disease status. In addition, the reproducibility of a test, which can be estimated by the intraclass correlation coefficient (ICC), indicates whether a single measurement of a biomarker can reasonably represent long-term levels and whether its concentration is relatively stable within an individual over time [20,21]. Attentiveness to these statistical parameters is important in clinical decision-making.

The aims of the present study were (1) to assess the prevalence of CS in high-risk ambulatory patients attending a University Hospital in Buenos Aires (Argentina) by performing all first-line diagnostic tests (UFC, SAF_{23} and F_{dex}) for each subject as well as, for the first time, UFC_{22-23} and SAF_{dex}; (2) to validate the reproducibility of UFC, SAF_{23} and UFC_{22-23} in at-risk subjects and CS patients using a unique cortisol radioimmunoassay; and (3) to define the performance of the tests with the aim of identifying a practical and non-invasive diagnostic approach for CS in ambulatory subjects with high pretest probability.

2. Experimental methods

2.1. Study population

Outpatients attending a University Hospital in Buenos Aires (Argentina) were referred from primary care physicians to the Endocrine Unit over a period of 12 months. The study was carried out in 128 consecutive outpatients: 10 with uncontrolled diabetes mellitus type 1 (DM_1), 57 with uncontrolled diabetes mellitus type 2 (DM_2) (all diabetics with $HbA1_c > 9.0\%$), 40 with resistant hypertension of unknown aetiology and central obesity (HBP) (blood pressure > 160/100 mmHg; weight/hip ratio >0.8 in females and >0.9 in males) and 21 premenopausal women with osteoporosis (Z score < −2.0) (OS). All patients had a glomerular filtration rate $\geqslant$60.0 ml/min/1.73 m^2. DM_1 patients were taking insulin, and DM_2 patients were on at least one oral antidiabetic drug (metformin, glibenclamide, glypizide) or combined therapy (20%). All HBP patients were on two antihypertensive drugs (enalapril, amlodipine, losartan, irbesartan, nifedipine). Many patients were also taking omeprazole, pantoprazole, simvastatin, atorvastatin, clonazepam or alprazolam. OS patients were following an adequate nutrition plan (protein, calcium and vitamin D).

Control groups included 100 healthy volunteers (C) with a glomerular filtration rate $\geqslant$90.0 ml/min/1.73 m^2 and no endocrine disease and 23 patients with confirmed CS, diagnosed as previously described [17]. In 13 of the CS patients, Cushing's disease was confirmed by histological findings after transsphenoidal surgery and postoperative hypocortisolism, whereas 10 patients exhibited adrenal CS (five with adenoma, three with carcinoma, one with primary pigmented nodular adrenal disease and one with ACTH-independent macronodular adrenal hyperplasia). All patients underwent adrenal surgery, with biochemical and clinical remission in seven cases and death in three cases.

All participants had no history of alcohol abuse and were free of exogenous glucocorticoids for at least 3 months before the study.

The following protocol was approved by the local ethical committee (IDIM A. Lanari, University of Buenos Aires), and all participants provided written consent.

2.2. Temporal study design

2.2.1. Total urine collection

Urine was collected for a 24-h period starting at 08.00 h for the assessment of total urinary cortisol (UFC) and creatinine levels.

2.2.2. One-hour urinary collection (UFC_{22-23})

Urine was collected during a 1-h period (22.00–23.00 h). The subjects emptied their bladders at the beginning of the collection period (22.00 h) and 1 h later collected specimen as previously described [15]. Cortisol and creatinine levels were assessed in this sample, which was obtained immediately before the nocturnal saliva collection.

2.2.3. Saliva collection

Saliva samples were obtained after confirming the integrity of salivary gland function as previously described [22]. Whole saliva was collected by directly spitting in sterile polypropylene tubes. Subjects were instructed not to brush their teeth but rather to rinse their mouths with tap water 2 h before saliva collection. Samples were obtained at 23.00 h, at least 2-h after the last meal. Exercise, tobacco, social drugs and alcohol consumption were not permitted before sampling. Once obtained, saliva samples were frozen until delivery to the laboratory.

Subjects in the study population and CS patients obtained basal samples of saliva and urine on two non-consecutive days (48-h interval) to assess the reproducibility of UFC, UFC_{22-23} and SAF_{23}.

2.2.4. Low-dose dexamethasone suppression test (1-mg DST)

At 23.00 h, 1 mg of dexamethasone was taken orally. The following day (at 08.00 h), samples of whole saliva and serum were obtained to measure cortisol levels (SAF_{dex} and F_{dex}, respectively). After centrifugation (1000*g* for 10 min), the supernatants were stored at −20 °C for further steroid analysis.

2.2.5. Longer low-dose dexamethasone suppression test (2-mg DST)

Serum and saliva samples for measuring cortisol levels were obtained after subjects took 0.5 mg of oral dexamethasone every 6 h for a 48-h period. The criteria used to define a normal cortisol level after 2-mg DST (Endocrine Research Laboratory) were as follows: total serum cortisol (F_{dex2mg}) $\leqslant$ 40.0 nM and morning salivary cortisol (SAF_{dex2mg}) $\leqslant$ 1.5 nM.

Fig. 1 summarises the day by day schedule of the protocol performed with the study population and patients with CS. Controls also followed the described schedule, obtaining samples on days 1, 4 and 8.

2.3. Hormone assays

2.3.1. Salivary cortisol

SAF was measured in saliva samples by RIA (Diagnostic Products Corporation, Los Angeles, CA, USA) as previously described [23]. SAF was expressed as nM, and the minimal detectable SAF concentration was 0.5 nM. SAF intra- and interassay coefficients of variation (CVs) were less than 6.0% and 13.0%, respectively.

2.3.2. Serum cortisol

This parameter was determined by RIA using a coat-a-count kit as described by the manufacturer (Diagnostic Products Corporation, Los Angeles, CA). The minimal detectable concentration was 6.0 nM. The intra- and interassay CVs were less than 5.0% and 6.0%, respectively.

2.3.3. Urinary cortisol

UFC was determined by a RIA coat-a-count kit as described by the manufacturer (Diagnostic Products Corporation, Los Angeles, CA) after the extraction of 500 μl of urine with 1.0 ml of dichloromethane. The minimal detectable concentration was 6.0 nM. The intra- and interassay CVs were less than 7.0% and 8.0%, respectively. The recovery test was 91.0–100.0%. Total urinary cortisol was expressed as nM/day, and UFC_{22-23} as ng/mg creatinine.

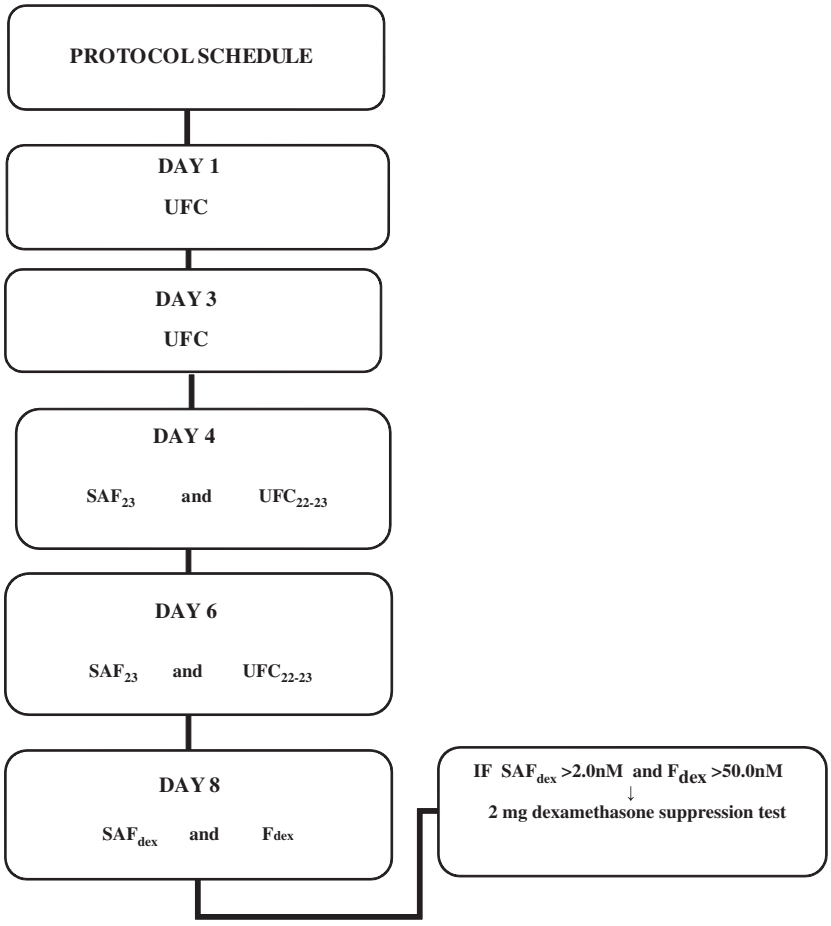

Fig. 1. Day by day schedule of the protocol performed in the study population.

Urine creatinine, plasma ACTH and serum DHEAS levels were measured by standard procedures in clinical chemistry laboratories as part of the patients' regular monitoring. Reference values (range) were as follows: urine creatinine: 13.0–25.0 mg creatinine/kg/day; ACTH: 2.2–11.0 pM; DHEAS: premenopausal women: 2.9–6.2 μM and postmenopausal women 2.0–4.4 μM.

2.4. Statistical analysis

Data are expressed as the mean ± SD unless otherwise specified. The variance component and the intraclass coefficient of correlation (ICCs) were estimated by a random-effects ANOVA model using the Statistical Package for Social Sciences (SPSS 11.5, SPSS Inc., Chicago, IL, USA). The ICC is ideally close to 1.0. The diagnostic performance of each test was evaluated by receiving operating curve analysis (ROC). The area under the curve (AUC_{ROC}) was defined, and threshold values were optimised for sensitivity. The spearman rank order test was used to estimate correlations between cortisol concentrations in different fluids; $p < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical features and cortisol measurements in 126 at-risk subjects in whom Cushing's syndrome was ruled out (non-CS)

The age, sex, BMI and cortisol dynamics of 100 healthy volunteers and 126 non-CS patients (10 DM_1, 56 DM_2, 39 HBP and 21 OS) are detailed in Table 1. The mean ages of DM_2 and OS patients were significantly higher than that of controls. The female/male ratio was $\geqslant 50$ in all groups. Control, DM_1 and OS subjects were mostly normoweight, while overweight and obese subjects were included in the DM_2 and HBP groups. Among the HBP patients, both UFC and UFC_{22-23} were significantly higher than in controls, whereas only UFC_{22-23} was significantly higher in the DM_2 group. However, for these patients, individual values of UFC and UFC_{22-23} were still below the threshold that excludes Cushing's syndrome.

Table 1 shows the good reproducibility of the UFC, UFC_{22-23} and SAF_{23} measurements obtained from two non-consecutive steroid samples in non-CS subjects ($\geqslant 0.870$). The ICCs of each steroid assay were as follows: UFC = 0.870, $UFC_{22-23} = 0.920$ and $SAF_{23} = 0.931$. Therefore, the within-subject variation was equal to or less than 13.0%. In all groups (C and non-CS), a positive and significant correlation was observed between UFC_{22-23} and SAF_{23} ($r = 0.718$ and 0.702, respectively), UFC_{22-23} and UFC ($r = 0.530$ and 0.540, respectively) and SAF_{23} and UFC ($r = 0.530$ for both) ($p \leqslant 0.0001$ in all cases). In all non-CS patients, SAF_{dex} and F_{dex} were positively and significantly correlated ($r = 0.781$; $p \leqslant 0.0001$). SAF_{dex} and F_{dex} values were higher in the DM_2 and HBP groups due to five DM_2 and one HBP patient not achieving the suppression threshold (Fig. 2 a and b, respectively). A 2-mg DST test was therefore performed in these patients, all of whom suppressed normally ($SAF_{dex\ 2\text{-}mg}$: 0.91 ± 0.30 nM and serum $F_{dex\ 2\text{-}mg}$: 17.5 ± 7.6 nM), confirming the false positive results of the 1 mg-DST test. In summary, six out of 126 patients required further testing with 2-mg DST to rule out CS.

Table 1
Clinical features and cortisol measurements in healthy subjects and at-risk patients in whom Cushing's syndrome was excluded.

	C	DM_1	DM_2	HBP	OS
n	100	10	56	39	21
Age (years)	30.0 ± 11.0(20.0–60.0)	34.0 ± 14.0 (17.0–58.0)	41.6 ± 12.7(20.0–60.0)**	39.0 ± 14.0(20.0–60.0)	44.0 ± 3(40.0–49.0)*
Female/male (n)	50/50	50/50	33/23	22/17	21
BMI (kg/m^2)	22.0 ± 1.8(18.5–24.9)	22.8 ± 1.5(21.0–24.9)	30.8 ± 2.6(26.0–35.0)**	33.0 ± 1.7(30.0–35.0)**	21.0 ± 2.0(18.5–24.9)
UFC (nmol/L)	96.0 ± 47.0(41.0–248.0)				
First sample		102.7 ± 41.0(43.0–171.0	104.0 ± 50.0(41.0–222.0)	115.0 ± 50.0(41.0–210.0)*	82.4 ± 31.0(43.0–162.0)
Second sample		121.0 ± 52.0(54.0–226.0)	104.0 ± 45.0(43.0–216)	117.0 ± 49.0 (40.0–217.0)*	90.0 ± 33.0(44.0–152.0)
$UFC_{22–23}$ (ng/mg cr.)	12.5 ± 11.0(0.1–44.0)				
First sample		19.8 ± 8.9(8.0–35.0)	17.7 ± 11.7 (0.5–44.0)*	19.3 ± 11.5 (15.5–44.0)**	16.0 ± 10.3(2.0–40.0)
Second sample		18.9 ± 8.0(8.0–36.0)	18.5 ± 11.4(0.5–44.0)*	19.8 ± 9.5 (4.7–40.0)**	17.4 ± 10.4(4.0–42.0)
SAF_{23} (nmol/L)	1.97 ± 0.96(0.5–3.8)				
First sample		1.35 ± 0.96(0.5–2.8)	1.81 ± 0.84(0.5–3.8)	2.0 ± 0.79 (0.5–3.6)	1.22 ± 0.64(0.5–2,7)**
Second sample		1.43 ± 0.90(0.5–2.8)	1.85 ± 0.85(0.5–3.8)	2.0 ± 0.72 (0.5–3.3)	1.29 ± 0.73(0.5–3.0)**
1-mg DST					
SAF_{dex} (nmol/L)	1.21 ± 0.51 (0.5–2.0)	1.26 ± 0.64 (0.5–2.0)	1.46 ± 0.98 (0.5–7.0)§	1.41 ± 0.50 (0.5–3.0)§	1.0 ± 0.50 (0.5–2.0)
F_{dex} (nmol/L)	26.0 ± 13.0 (13.8–50.0)	28.8 ± 14.3 (13.8–49.0)	42.3 ± 42.0 (14.0–331.0)§§	36.6 ± 19.7 (13.8–132.0)§	26.7 ± 12.9 (13.8–49.0)

Abbreviations: C: healthy subjects, DM_1: type-1 diabetes mellitus, DM_2: type-2 diabetes mellitus, HBP: hypertension and obesity, OS: osteoporosis, BMI: body mass index, UFC: total urinary free cortisol, $UFC_{22–23}$: 22.0–23.00 h urinary cortisol, SAF_{23}: salivary cortisol obtained at 23.00 h. First and second samples for UFC, $UFC_{22–23}$ and SAF_{23} were obtained in non consecutive days within the same week (except C). 1-mg DST: 1 mg dexamethasone suppression test with assessment of morning salivary cortisol (SAF_{dex}) and serum cortisol (F_{dex}). Data are expressed as mean ± SD (range).
Basal measurements:
*$P \leqslant 0.05$ versus C,
**$P \leqslant 0.001$ vs. C. 1-mg DST:
§$P \leqslant 0.05$ versus C;
§§$P \leqslant 0.001$ versus C.

3.2. Diagnosis of CS in two at risk subjects

Individual clinical and biochemical data are shown in Table 2. Case 1 was a postmenopausal woman who had gained 30 pounds over the previous 4 years. She had poor glycaemic control on oral hypoglycaemic agents and insulin as well as hyperlipemia and uncontrolled hypertension. Fatigue progressed over time, making her unable to work as a seamstress. She had a mild dorsocervical fat pad.

Suspicion of cortisol excess was confirmed by the elevation of at least two basal measurements ($UFC_{22–23}$ and SAF_{23}) and an unsuppressed cortisol level after 1 mg DST treatment. UFC was either normal or moderately elevated in different samples. Unsuppressed ACTH levels revealed ACTH dependence. A hypodense intrasellar MRI image prompted a surgical exploration of the pituitary, and an ACTH-secreting microadenoma was resected.

Postoperatively, hypocortisolism was confirmed by low levels of morning serum cortisol on two consecutive days (27.5 nM and 41.0 nM). Upon tapering the oral substitute hydrocortisone, recovery of adrenal cortisol function was followed by stimulating the adrenal cortex by intramuscular administration of a low dose of ACTH [23] every three months. At present, 12 months after surgery, the patient is taking 5 mg of hydrocortisone/day and has good glycaemic control on metformin, and her blood pressure is stable on amlodipin. She has resumed her daily work.

Case 2 was a premenopausal woman with the following major complaints: central obesity, resistant hypertension, fatigue and oligomenorrhea for approximately 2 years. She was a high school

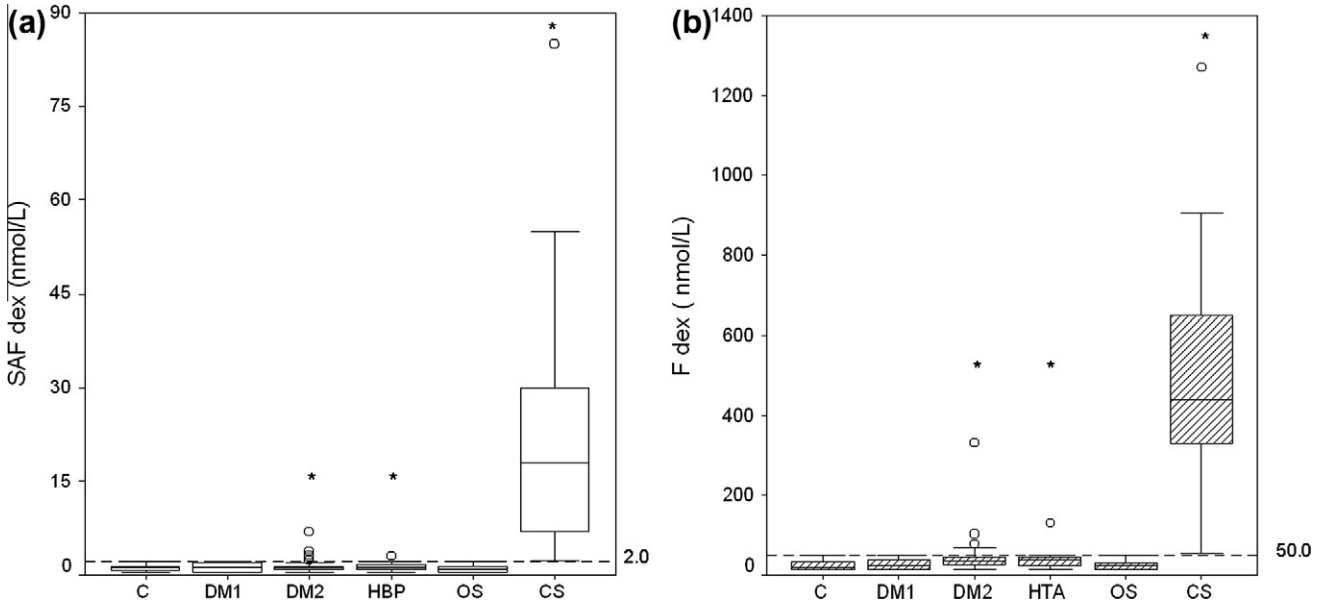

Fig. 2. Box and whisker plots show (a) salivary cortisol (SAF_{dex}) and (b) serum cortisol (F_{dex}) at 08.00 h after 1-mg DST treatment in healthy volunteers, at-risk patients and patients with Cushing's syndrome. C: healthy subjects; DM_1: type 1 diabetes mellitus; DM_2: type 2 diabetes mellitus; HBP: hypertension and obesity; OS: osteoporosis; CS: Cushing's syndrome. The horizontal line represents the median, the box indicates the interquartile range, and the whiskers show the date range, excluding the indicated outliers. The horizontal dotted lines indicate the upper limit of the reference range. *$p \leqslant 0.05$ *vs.* controls.

Table 2
Diagnosis of Cushing's syndrome in two women out of 128 at-risk subjects.

	Patients	
	Case 1	Case 2
Age (years)	53.0	35.0
BMI (kg/m^2)	30.8	30.0
UFC (nmol/L)		
First sample	363.0	256.0
Second sample	240.0	250.0
UFC_{22-23} (ng/mg cr)		
First sample	112.0	66.0
Second sample	113.0	56.0
SAF_{23} (nmol/L)		
First sample	4.5	13.0
Second sample	5.0	12.0
1-mg DST		
SAF_{dex} (nmol/L)	7.0	6.0
Fdex (nmol/L)	414.0	275.0
ACTH pl (pmol/L) (mean ± SD)	4.4 ± 0.9	6.0 ± 0.5
DHEA-S (μmol/L)	3.2 ± 0.6	4.5 ± 0.5
MRI sellar	Hypodense image (size 10.0mm)	Normal
BIPSS	Not done	Positive
Adrenal CT	Not done	Not done
Hystological confirmation	Pituitary ACTH-secreting microadenoma	PituitaryACTH-secreting microadenoma

Abbreviations: BMI: body mass index, UFC: total urinary free cortisol, UFC_{22-23}: 22.0–23.00 urinary cortisol, SAF_{23}: salivary cortisol obtained at 23.00 h. First and second samples were obtained in non consecutive days within the same week. 1-mg DST: 1 mg dexamethasone suppression test with assessment of morning salivary cortisol (SAF_{dex}) and serum cortisol (F_{dex}); DHEA-S: dehydroepiandrosterone sulfate; MRI: magnetic resonance image; BIPSS: bilateral inferior petrosal sinus sampling; Adrenal CT: adrenal computed tomography. Reference values: see Materials and methods.

gym teacher but had been off work for elevated blood pressure and increased fatigue. UFC was assessed on suspicion of CS, and slightly elevated levels were observed on two separate occasions. The loss of the nocturnal cortisol nadir (high UFC_{22-23} and SAF_{23}) and the lack of cortisol suppression after 1-mg DST treatment strongly supported the diagnosis of CS. Normal ACTH levels suggested the pituitary dependence of the syndrome. Because the sellar MRI was unremarkable, a bilateral inferior petrosal sinus sampling was performed, and central ACTH production was confirmed. The patient therefore underwent transphenoidal pituitary exploration, during which an 8 mm pituitary adenoma was excised. Postoperative hypocortisolism was observed on two consecutive days (serum cortisol: 27.5 nM and 30.0 nM), and the patient was started on replacement doses of hydrocortisone. Recovery of adrenal function was assessed every 3 months. Persistent hypoadrenal function on hydrocortisone 10 mg/day has been observed to date (9 months after surgery). The patient experienced an improvement in mood and fatigue, achieved normotension and returned to work.

3.3. Clinical and biochemical data in patients with CS: comparison with healthy subjects

Table 3 describes the clinical and laboratory data of previously diagnosed CS patients combined with the newly diagnosed CS patients (cases 1 and 2). CS patients were significantly older and heavier than controls. UFC, UFC_{22-23} and SAF_{23} were statistically higher in CS patients than controls.

The reproducibility of UFC (ICC = 0.80), UFC_{22-23} (ICC = 0.98) and SAF_{23} (ICC = 0.95) in CS patients was quite good. In addition, UFC, UFC_{22-23} and SAF_{23} were positively and significantly correlated ($r \geqslant 0.516$; $p \leqslant 0.001$ in all).

SAF_{dex} and F_{dex} were significantly correlated after 1-mg DST therapy ($r = 0.890$; $p \leqslant 0.0001$) and higher than in controls ($p \leqslant 0.001$), revealing an impairment of normal suppression (Fig. 2a and b).

Table 3
Clinical and laboratory data in 25 patients with Cushing's syndrome.

	C	CS
n	100	25
Age (years)	30.0 ± 11.0(20.0–60.0)	41.2 ± 10.9 (20.0–55.0)**
Female/male (%)	50.0/50.0 (50.0/50.0)	20/5 (80.0/20.0)
BMI (kg/m2)	22.0 ± 1.8(18.5–24.9)	28.4 ± 1.6(25.0–30.0)**
UFC (nmol/L)	96.0 ± 47.0(41.0–248.0)	
First sample		666.0 ± 439.0 (143.0–1793.0)**
Second sample		635.0 ± 432.0 (178.0–1925.0)**
ICC coefficient		0.80
UFC_{22-23} (ng/mg cr.)	12.5 ± 11.0(0.1–44.0)	
First sample		238.0 ± 196.0(60.0–700.0)**
Second sample		239.0 ± 195.0 (56.0–670.0)**
ICC coefficient		0.98
SAF_{23} (nmol/L)	1.97 ± 0.96(0.5–3.8)	
First sample		22.8 ± 23.8(4.5–100.0)
Second sample		22.0 ± 21.7(4.5–80.0)
ICC coefficient		0.95
Spearman's rho		
SAF_{23} *vs.* UFC_{22-23}	r :0. 718‡	r :0.775‡
SAF_{23} *vs.* UFC	r :0. 530‡	r :0.516‡
UFC_{22-23} *vs.* UFC	r :0. 530‡	r :0.550‡
1-mg DST		
SAF_{dex} (nmol/L)	1.21 ± 0.51(0.5–2.0)	22.0 ± 19.7(2.2–85.0) §
F_{dex} (nmol/L)	26.0 ± 13.0(13.8–50.0)	500.0 ± 252.0(55.0–1272.0) §

Abbreviations: C: healthy subjects, CS: patients with Cushing's syndrome; UFC: total urinary free cortisol, UFC_{22-23}: 22.0–23.00 urinary cortisol, SAF_{23}: salivary cortisol obtained at 23.00 h. First and second samples were obtained by CS patients in non consecutive days within a week. 1-mg DST: 1 mg dexamethasone suppression test with assessment of morning salivary cortisol (SAF_{dex}) and serum cortisol (F_{dex}); ICC: intraclass correlation coefficient; Spearman's rho: correlation test. Data are expressed as mean ± SD (range).
Statistical significance.
** $P \leqslant 0.001$ *vs.* control group.
Rho correlation:
‡ $P \leqslant 0.001$.
1-mg DST:
§ $P \leqslant 0.001$ *vs.* C.

Table 4
Performances of diagnostic tests for endogenous hypercorticism.

Test (cut-off value)	Sensitivity [%(95%CI)]	Specificity [%(95%CI)]	AUC_{ROC} (95%CI)	Positive predictive value	Negative predictive value
24 h urinary free cortisol (>248.0 nmol/d)	94.0 (83.4–98.7)	100.0 (98.9–100.0)	0.994 (0.980–0.990)	100.0	99.0
22.0–23.0 h urinary cortisol (>44.0 ng/mg creatinine)	100.0 (92.8–100.0)	100.0 (99.0–100.0)	1.000 (0.990–1.000)	100.0	100.0
23.0 h salivary cortisol (>3.8 nM)	100.0(92.8–100.0)	100.0 (98.9–100.0)	1.000(0.990–1.000)	100.0	100.0
Post 1 mg dexamethasone salivary cortisol (>2.0 nM)	100.0 (86.2–100.0)	97.3 (94.3–99.0)	0.998 (0.981–0.999)	80.6	100.0
Post 1 mg dexamethasone serum cortisol (>50.0 nM)	100.0 (86.2–100.0)	97.3 (94.3–99.0)	0.998 (0.981–0.999)	80.6	100.0

The cut-off values are estimated by ROC analysis and optimized for sensitivity.

3.4. Accuracy of diagnostic tests for endogenous hypercortisolism

We performed an ROC curve analysis using data from 226 eucortisolemic subjects (non-CS and healthy controls) and 25 patients with endogenous hypercortisolism (CS). The analysis revealed that under basal conditions, late-night cortisol measurements of $UFC_{22–23}$ or SAF_{23} show 100% sensitivity and specificity compared with cortisol levels assessed in 24-h urine samples. Supressed cortisol in serum or saliva showed 100% sensitivity with 97.3% specificity (Table 4)

4. Discussion

This study is the first to attempt a comprehensive diagnostic work-up to assess CS in at-risk subjects. In each case, every first-line diagnostic test recommended for CS was applied, with the addition of assays for 22.00–23.00 h urinary cortisol and salivary cortisol after 1-mg DST. CS was diagnosed in two at-risk subjects (1.5%) out of 128 ambulatory subjects. Postsurgical hypocortisolism was considered the gold standard clinical criterion to confirm the diagnosis of CS. ACTH-dependent CS was demonstrated in two women with central obesity and poor metabolic control of diabetes mellitus type 2 (case 1) or resistant arterial hypertension (case 2). Postoperatively (9–12 months), both patients had experienced significant reductions in body weight and waist circumference, improved glycaemic control (case 1) and either ameliorated or normalised arterial blood pressure (cases 1 and 2, respectively).

A lack of circadian rhythm was observed in the cortisol levels of ACTH-dependent CS patients, in association with a variable daily cortisol production. Consistently, both diagnosed patients showed unsuppressed cortisol levels in serum and saliva after 1-mg DST therapy, indicative of impairment in the negative feedback mechanism. In CS patients, the UFC reproducibility (ICC = 0.80) displayed higher within-person variability than $UFC_{22–23}$ or SAF_{23} (ICC $\geqslant$ 0.95, for both), making UFC comparably less reliable when measuring single samples. ROC analysis demonstrated that late-night cortisol levels in 1 h-urine or saliva samples were 100% sensitive and specific for CS, while suppression of cortisol (either in saliva or serum) after 1-mg DST treatment showed 100% sensitivity but lower specificity (97.3%).

In our study, CS was detected among patients lacking specific signs of hypercortisolism but with morbidities such as uncontrolled diabetes mellitus type 2 and resistant arterial hypertension of unknown aetiology associated with central obesity, in agreement with previous findings [1,3,6,7,9,24]. However, CS was not detected among either premenopausal women with idiopathic osteoporosis or in patients with type 1 diabetes mellitus, as reported by others [2,8]. Interestingly, moderate osteopenia (*T* scores between −1.5 and −2.0), but not osteoporosis, was observed in CS women. This worrisome clinical scenario might be ascribed to glucocorticoid sensitivity, which may differ among individuals, between tissues of the same individual or as a consequence of polymorphisms in the genes encoding the glucocorticoid receptor and 11-beta-hydroxysteroid-dehydrogenase type 1 [25,26]. The subtle clinical features of excess cortisol in CS patients might therefore be modulated by variability in cortisol secretion or peripheral cortisol sensitivity [5].

Endogenous hypercortisolism, even when mild, is physically and emotionally harmful. Early diagnosis and effective treatment in the two newly diagnosed patients resulted in improvement of glycaemic control, arterial blood pressure and obesity, as previously reported [1,2,9]. Quality of life, as assessed by a SF-36 health survey questionnaire administered before and six months after surgery, reflected better vitality, social function and mental health (data not shown).

Common sources of error and bias in biomarker studies include issues related to laboratory assays, specimen collection and storage and within-person variability over time. Radioimmunoassays are widely used in the analysis of steroids because of their simplicity, speed, sensitivity and low cost. In this study, we used a coat-a-count RIA to assay cortisol in all biofluids. To reduce preanalytical errors related to this technique (e.g., antibody cross-reactivity), all samples were processed with the same antibody and tracer. Sample collection instructions were explained to individual patients by physicians or qualified laboratory operators.

To our knowledge, this is the first study to measure the correlation between UFC, UFC_{22-23} and SAF_{23} in healthy subjects ($n = 100$), at-risk individuals in whom CS was ruled out ($n = 126$) and patients with confirmed CS ($n = 25$). These correlations were positive and significant in all cases ($r > 0.51$; $p \leqslant 0.001$).

ICC is a good measure of reproducibility, as it takes into account both between- and within-person variability. An ICC $\geqslant 0.75$ indicates excellent reproducibility [27]. The ICCs of UFC, UFC_{22-23} and SAF_{23} in non-CS and CS patients were all $\geqslant 0.80$, as we previously reported [9,17], in agreement with others [28,29].

Our ROC analysis showed that SAF_{23} and UFC_{22-23} at threshold values >3.8 nM and >44.0 ng/mg creatinine, respectively, shared the highest sensitivity and specificity for detecting the absence of the cortisol nadir. The threshold values of suppressed serum cortisol ($\leqslant$50.0 nM) obtained in this study are in agreement with data based on compilation studies [10]. The suppressed salivary cortisol threshold value ($\leqslant$2.0 nM) confirmed our own previous report [17]. An absence of cortisol suppression in serum and saliva after 1-mg DST therapy was demonstrated in six obese non-CS patients (five DM_2 and one HBP), but suppression was achieved after 2-mg DST treatment, as previously described in obese subjects as well as patients on multiple drugs that may interfere with dexamethasone metabolism [9,30]. Thus, the 1-mg DST test for suppressed cortisol in saliva or serum had a weaker positive predictive value.

The limitations of this study include the small number of patients with osteoporosis and uncontrolled diabetes mellitus type-1, the absence of a comparison group of individuals suspected of having CS, the lack of follow-up among patients with negative tests to detect very rare cases of cyclical CS and the uncertain reproducibility of the tests in cases of longer interval sampling.

In summary, single samples of UFC_{22-23} or SAF_{23} can be interchangeably used to non-invasively screen subjects with a high pre-test probability of CS. These assays may serve as helpful clinical tools for patients presenting with conditions that invalidate saliva or urine sampling, such as bleeding gums, Sjöegren syndrome or renal failure.

The assessment of UFC_{22-23} or SAF_{23} with SAF_{dex} offers a non-invasive and accurate diagnostic tool for the evaluation of the cortisol nadir and feedback status in ambulatory at-risk populations.

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