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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.
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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.

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