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ORIGINAL ARTICLE

Diagnostic value of salivary cortisol in Cushing's syndrome (CS)

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Summary

Objective The diagnosis of Cushing's syndrome (CS) remains a challenge in clinical endocrinology. The aim of this study was to determine the reproducibility and diagnostic value of late-night salivary cortisol (SAF_{23}) for CS and its utility along the follow-up of treated patients. In addition, using the same radioimmunoassay reactives, the cut-off values for saliva and serum cortisol, assessed synchronically after the overnight 1 mg dexamethasone suppression test (DST), were defined.

Design Twenty-one patients with confirmed CS and 121 volunteers were studied. All the subjects collected 24-h urine for cortisol (UFC). On the same day whole saliva was obtained from the subjects at 23 h for SAF_{23}. The intraclass coefficient of correlation (ICC) of SAF_{23} was estimated in 47 subjects (21 CS and 26 C). At 8 h, after DST, simultaneous saliva and serum samples for cortisol (SAF_{dex} and F_{dex}, respectively) were obtained in 51 subjects (17 CS and 34 C). After specific therapy, 18 patients with CS were followed with SAF_{23} measurements. SAF and F were expressed as nM.

Results The intraclass coefficient of correlation of SAF_{23} was 0·89 in CS and 0·83 in C. $SAF_{23} > 3{\cdot}8$ nM showed a sensitivity and specificity of 100% and 97·5%, respectively, for diagnosing CS. SAF_{23} correlated positively with UFC ($r = 0{\cdot}685$; $P = 0{\cdot}0001$). After DST, SAF_{dex} significantly correlated with F_{dex} ($r = 0{\cdot}61$, $P < 0{\cdot}0001$). A cut-off value of $SAF_{dex} > 2{\cdot}0$ nM and $F_{dex} > 50{\cdot}0$ nM detected CS with 100% sensitivity and specificity. After successful surgical therapy, 13 patients with CS had SAF_{23} levels < 3·8 nM ($1{\cdot}4 \pm 0{\cdot}8$ nM).

Conclusions SAF_{23} and SAF_{dex} seem to be good screening tools based on their noninvasive nature, remarkable reproducibility and diagnostic performances.

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Introduction

The diagnosis of spontaneous Cushing's syndrome (CS) remains a challenge in clinical endocrinology. Since long ago clinicians have been looking for a biochemical steroid marker that combines practicity with high sensitivity for the detection of this disorder.

Total urinary free cortisol (UFC) provides an integrated assessment of cortisol secretion over a 24-h period, but a complete day collection with appropriate total volume in accordance with urinary creatinine levels are required for an accurate diagnosis of cortisol excess.[1]

The lack of circadian rhythm of cortisol is the hallmark of CS, whatever is its cause. Several screening tests (cortisol in spot urine, serum and saliva) have been proposed to detect this abnormality.[2–4] However, although reliable to rule out CS, some of these tests are not currently used (spot urine) or may be troublesome (midnight serum samples need patients' hospitalization). Thus, salivary cortisol, which reflects the free fraction of total serum cortisol, becomes a safe and practical alternative to reveal hypothalamic–pituitary–adrenal (HPA) axis disarrangements when assessed late at night.[4,5]

Late-night salivary cortisol has been used recently by many centres as a first line diagnostic test for CS, yet its accuracy is still on debate.[4–9] However, experts have recommended the publication of data from individuals free of disease and patients with confirmed CS to define its diagnostic accuracy.[9] For this purpose, each laboratory should validate its own methodology and be aware of sampling conditions to get reliable data.[10–12]

Failure of normal suppression of serum cortisol after 1 mg dexamethasone suppression test (DST) is a well-known biochemical screening test when CS is suspected, showing a sensitivity and specificity > 95% and 80%, respectively.[1] Considering that basal salivary cortisol correlates positively with total serum cortisol when proteins are normal and samples are synchronically obtained,[6,13,14] measurement of salivary cortisol after DST (SAF_{dex}) was proposed to simplify this test. This modality turns DST completely ambulatory and more practical. Available data on SAF_{dex} for the screening of adult patients with CS are scarce[15–18] and most of them do not describe the performance of the simultaneous assessment of cortisol in saliva and serum. Thus, the diagnostic value of salivary cortisol in DST remains unclear.

Since 2000, we focused our research on the application of salivary cortisol as a valuable alternative to blood-borne analysis in the assessment of HPA axis.

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The aims of this study were: (i) to validate the reproducibility of late-night salivary cortisol (SAF_{23}) in patients with CS; (ii) to determine the diagnostic value of SAF_{23} for CS; (iii) to define our own cut-off values for salivary and serum cortisol when assessed synchronically in DST using the same radioimmunoassay (RIA) reactives; and (iv) to assess the utility of SAF_{23} in the follow-up of treated patients.

Subjects and methods

Twenty-one subjects with confirmed CS were studied [17 female and 4 male, aged 24–55 years, body mass index (BMI) = median: 26 kg/m^2, range: 22·8–27 kg/m^2]. They were all diagnosed for CS according to the usual clinical and biochemical features with at least two abnormal first line diagnostic tests (UFC, SAF_{23} or DST).[7] Among these 21 patients, 11 had Cushing's disease (CD) confirmed by histological findings after transsphenoidal surgery in 10 cases (selective adenomectomy and total hypophysectomy in eight and two cases, respectively), because one CD patient achieved spontaneous remission. Nine patients had adrenal CS (five with adenoma, two with carcinoma, one with primary pigmented nodular adrenal disease and one with ACTH-independent macronodular adrenal hyperplasia). All underwent adrenal surgery, with biochemical and clinical remission in seven cases and death in two cases (adrenal carcinoma). One patient had proven ectopic ACTH secretion due to an oat cell bronchial carcinoma and died 3 months after surgery. Patients were not taking drugs known to interfere with pituitary adrenal secretion or cortisol measurements at the period of this study.

The control group (C) comprised 121 volunteers: 61 with normal weight (32 females, 29 males, aged 22–55 years, BMI = 22–25 kg/m^2) and 60 overweight (36 females, 24 males, aged 30–57 years, BMI = 26–28 kg/m^2). None of these subjects had endocrine pathology and were on no medication. Similar mean UFC levels were found among normal and overweight subjects (88·0 ± 45·0 and 99·0 ± 47·0 nmol/day, respectively, $P = 0{\cdot}113$). In addition, SAF_{23} levels were not different (2·00 ± 0·96 and 1·80 ± 0·95 nM, respectively; $P = 0{\cdot}157$). Albumin concentrations in CS and C were within the normal range (3·3–4·8 g/dl).

The following protocol study was approved by the Local Ethics Committee (School of Medicine, University of Buenos Aires). Informed consent was obtained from all the subjects.

Study design

Urine collection. Urine was collected for a 24-h period starting at 8 h for cortisol (UFC) and creatinine measurements.

Late-night saliva collection. On the day of urine collection, subjects were instructed to collect saliva for SAF_{23}. They had to avoid meals, alcohol, coffee, tea, mate, exercising, smoking and tooth brushing 2 h before sampling. At 23 h, subjects collected whole saliva by directly spitting in sterile polypropylene tubes. Once obtained, samples were frozen until delivery to the laboratory.

In 26 randomly selected healthy subjects and 21 patients with CS, nocturnal saliva was obtained in two nonconsecutive days (48-h interval) to assess the reproducibility of SAF_{23} assay.

Low-dose DST. Overnight DST was performed in 51 subjects (34 randomly selected healthy subjects and 17 patients with CS). At 23 h, 1 mg of dexamethasone was given orally and the following day (at 8 h) simultaneous whole saliva and serum samples for cortisol (SAF_{dex} and F_{dex}, respectively) were obtained. After centrifugation (1000 ***g***, 10 min) the supernatants of saliva and serum were kept at –20 °C for further analysis.

Late-night salivary cortisol measurements in the follow-up of treated patients. One week after surgery, late-night salivary cortisol and morning serum cortisol were assessed in 13 cases. Patients on adrenostatic drugs were followed monthly with SAF_{23} and UFC measurements.

Salivary cortisol assay. SAF was measured by RIA (Diagnostic Products Corporation, Los Angeles, CA) in saliva samples as previously described.[19] 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% and 13%, respectively.

Serum cortisol assay. F was determined by RIA using a coat-a-count kit as described by the manufacturer (Diagnostic Products Corporation). The minimal detectable dose was 6·0 nM. The intra- and interassay CVs were less than 5·0% and 6·0%, respectively.

Urinary cortisol assay. UFC was determined by a RIA coat-a-count kit after the extraction of 500 µl of urine with 1·0 ml of dichloromethane as described by the manufacturer (Diagnostic Products Corporation). The minimal detectable dose was 6·0 nM. The intra- and interassay CVs were < 7·0% and 8·0%, respectively. The recovery test was 91·0–100·0%. The accuracy of 24-h urine collection was confirmed by measuring creatinine excretion.

Statistical analysis

Data are expressed as mean ± SD or otherwise specified. Variance components and intraclass coefficients of correlations (ICCs) were estimated by random-effects ANOVA model using the Statistical Package for the Social Sciences (SPSS 11·5, SPSS Inc, Chicago, IL). The ICC is an index of reproducibility for a test, being ideal close to 1·0 (close to 100% of the observed variation explained by between-individual variation). The diagnostic performance of the test was evaluated by the receiving operating curve (ROC) analysis obtained by Medical Calc (version 7·3·01; 2004). The area under the curve (AUC_{ROC}) was defined and cut-off values were optimized for sensitivity. When specified, a grey zone was defined as an 'area' of values where the discriminatory performance of the test is insufficient to allow the presence or absence of a disease to be scored.[20] Spearman's rank order test was used to check the correlation of cortisol concentrations in different fluids. Regression analysis between variables was obtained by Medical Calc. A $P < 0{\cdot}05$ was considered statistically significant.

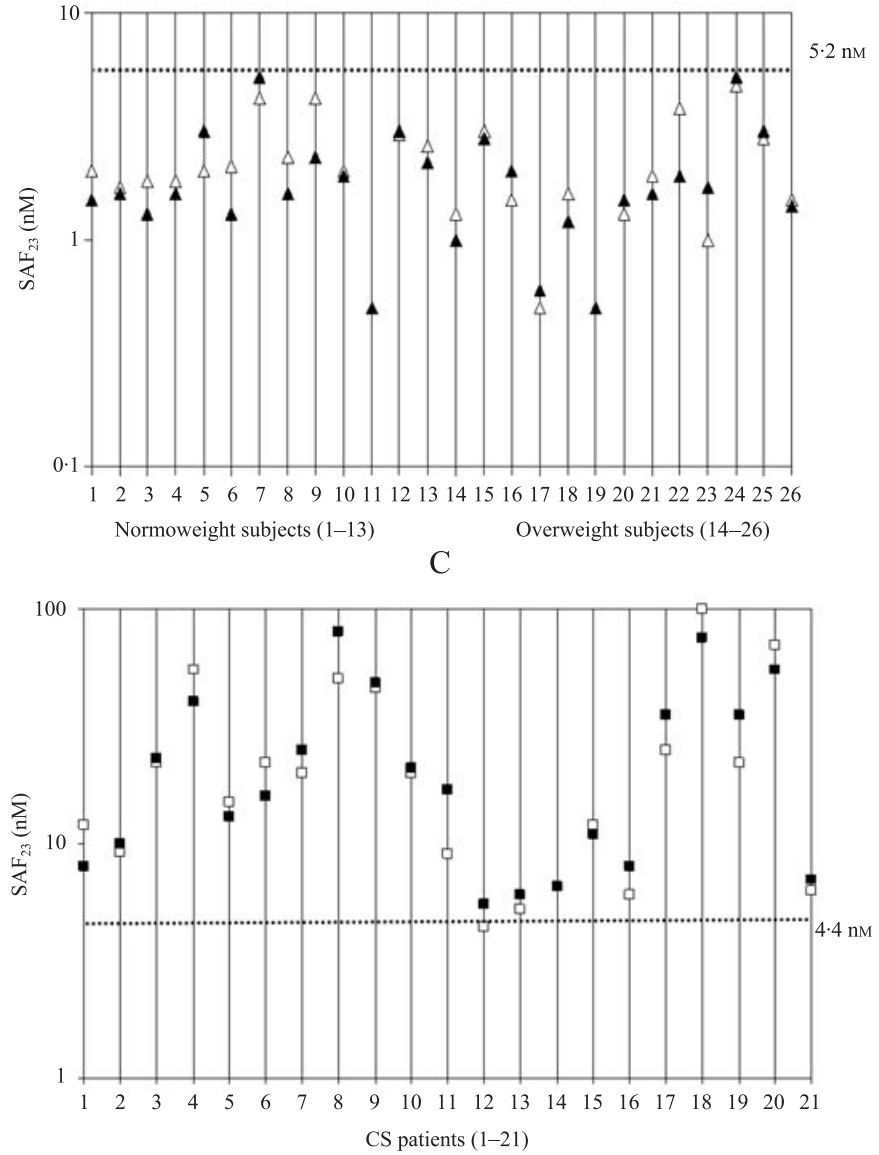

Fig. 1 Late-night salivary cortisol in samples obtained in two nonconsecutive days from healthy subjects (a) and patients with Cushing's syndrome (b). △ First sample; ▲ Second sample. Dotted lines show the maximum (a) and the minimum (b) values found in control (C) and Cushing's syndrome (CS), respectively.

Results

Reproducibility of late-night salivary cortisol measurements

Individual data of SAF_{23} (two different samples) are displayed in Fig. 1a,b. In healthy subjects, SAF_{23} values obtained from sequential samples (first sample = 2·14 ± 1·16 nM, second sample = 1·98 ± 1·18 nM) were not significantly different ($P = 0·605$) as observed in patients with CS (25·6 ± 25·0 *vs.* 25·9 ± 22·4 nM; $P = 0·962$). The calculated ICC was 0·83 and 0·89 for healthy subjects and patients with CS, respectively, that is, the variation of SAF_{23} was 17% or less in each subject.

Late-night salivary cortisol and total UFC in patients with CS

SAF_{23} levels (25·7 ± 24·9 nM) from 21 patients with CS were significantly higher than those found in 121 healthy subjects (1·91 ± 0·96 nM), $P = 0·0001$. Figure 2 plots individual SAF_{23} concentrations. The performance of SAF_{23} measurement ($n = 142$) for the biochemical diagnosis of CS was estimated by ROC analysis and displayed in Table 1. On the basis of these results, cut-off values for negative, grey zone and positive SAF_{23} results are < 3·8 nM, 4·4–5·2 nM and > 5·2 nM, respectively. Thus, $SAF_{23} > 3·8$ nM is able to diagnose CS with a sensitivity and specificity of 100% and 97·5%, respectively.

In patients with CS, UFC concentrations (800·0 ± 770·0 nmol/day; median: 442·0, range: 143·0–3576·0 nmol/day) were significantly higher than in healthy subjects (95·0 ± 47·0 nmol/day; median: 83·0, range: 41·0–248·0 nmol/day; $P = 0·0001$). Only one patient with CS had UFC levels (143·0 nmol/day) lower than the upper limit of the normal range (248·0 nmol/day); this patient had a mild form of CS. The other 20 patients had overt CS with UFC levels that ranged from 309·0 to 3576·0 nmol/day (25·0–1342·0% above the upper limit of the normal range). Figure 3 and Table 1 show that a threshold value of UFC > 248·0 nmol/day has a sensitivity and specificity of 95·2%

Table 1. Performances of diagnostic tests for Cushing's syndrome

Test (cut-off value)	Sensitivity [% (95% CI)]	Specificity [% (95% CI)]	AUC_{ROC} (95% CI)
Late-night salivary cortisol (> 3·8 nM)	100 (83·7–100·0)	97·5 (92·9–99·5)	0·999 (0·972–1·000)
24-h Urinary free cortisol (> 248·0 nmol/day)	95·2 (76·1–99·2)	100·0 (97·0–100·0)	0·992 (0·960–0·999)
Post-dexamethasone salivary cortisol (> 2·0 nM)	100·0 (80·3–100·0)	100·0 (89·6–100·0)	1·000 (0·930–1·000)
Post-dexamethasone serum cortisol (> 50·0 nM)	100·0 (80·3–100·0)	100·0 (89·6–100·0)	1·000 (0·930–1·000)

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

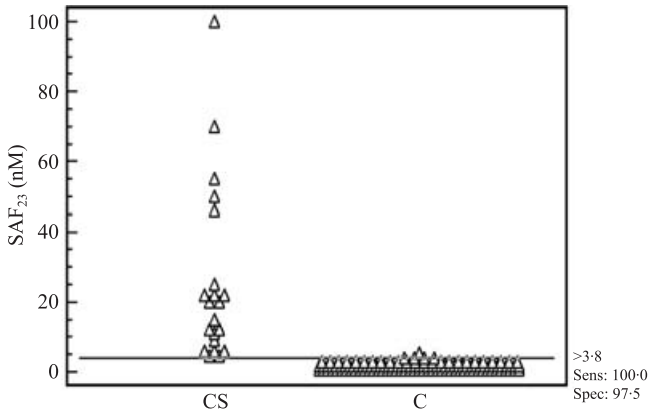

Fig. 2 Dot diagram of late-night salivary cortisol (SAF_{23}) levels obtained from healthy subjects (C) and patients with Cushing's syndrome (CS). A cut-off > 3·8 nM for SAF_{23} showed the best prediction accuracy for CS with 100% sensitivity and 97·8% specificity.

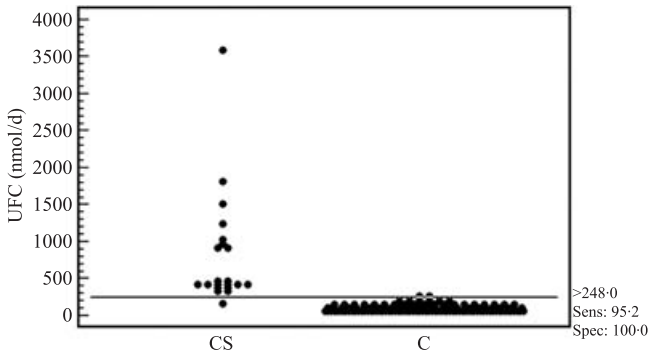

Fig. 3 Dot diagram of total urinary cortisol (UFC) levels obtained from healthy subjects (C) and patients with Cushing's syndrome (CS). A cut-off > 248·0 nmol/day for UFC showed 95·2% sensitivity and 100% specificity to detect CS.

and 100%, respectively, for the diagnosis of CS. UFC correlated positively with SAF_{23} ($r = 0{\cdot}685$; $P = 0{\cdot}0001$) in the whole population ($n = 142$).

Morning salivary and serum cortisol levels after overnight 1 mg oral dexamethasone in patients with CS

In patients with CS, SAF_{dex} (24·32 ± 26·0 nM) and F_{dex} (445·0 ± 292·0 nM) levels were significantly higher than in healthy subjects (1·10 ± 0·53

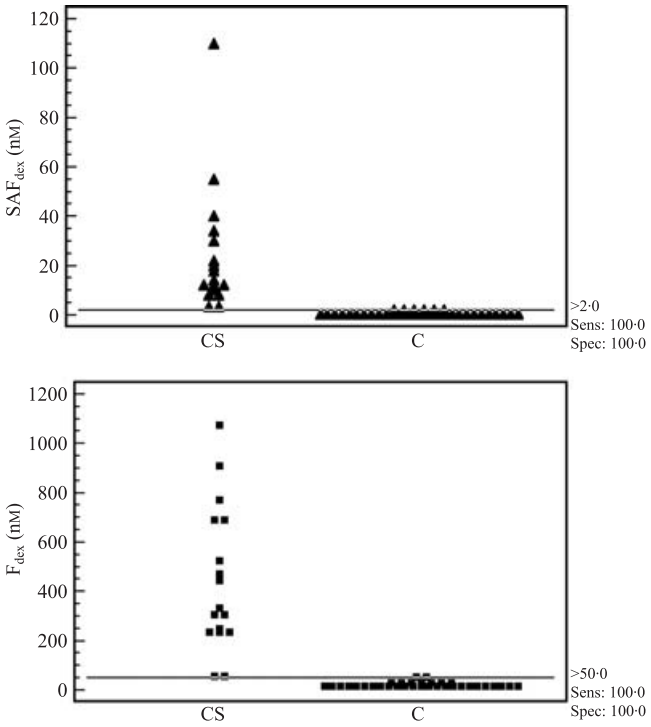

Fig. 4 Dot plot of salivary cortisol (SAF_{dex}) and serum total cortisol (F_{dex}) after dexamethasone suppression test (DST) in healthy subjects (C) and patients with Cushing's syndrome (CS). A positive test was defined as a SAF_{dex} > 2·0 nM (a) and F_{dex} > 50·0 nM (b) with optimal sensitivity and specificity.

and 19·7 ± 10·0 nM, respectively; $P = 0{\cdot}0001$). SAF_{dex} significantly correlated with F_{dex} in all the subjects ($r = 0{\cdot}61$, $P = 0{\cdot}0001$). The relationship between these two variables was represented by the regression equation $y = 63{\cdot}2 + 11{\cdot}10x$ (coefficient of determination = 0·612).

Cut-off values for SAF_{dex} > 2·0 nM (Fig. 4a) and F_{dex} > 50·0 nM (Fig. 4b) were able to diagnose CS with 100% sensitivity (Table 1).

Late-night salivary cortisol in the follow-up of treated patients with CS

Surgical therapy was successful in 13 patients: six with CD underwent transsphenoidal selective pituitary adenomectomy, five with adrenal adenomas had unilateral adrenalectomy, one with ACTH-independent macronodular adrenal hyperplasia and one primary pigmented nodular adrenal disease had bilateral adrenalectomy. One week after surgery, patients were re-evaluated off hydrocortisone,

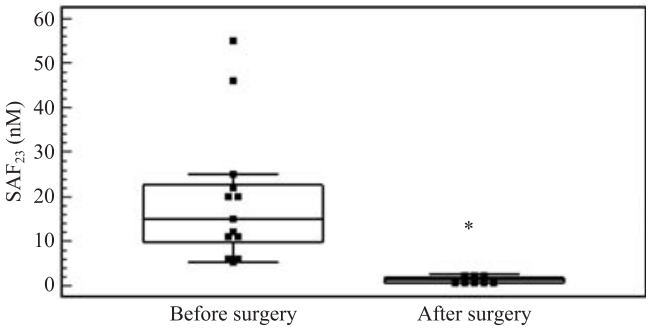

Fig. 5 Late-night salivary cortisol levels (SAF_{23}) from 13 patients with active Cushing's syndrome, before and after surgical remission of hypercortisolism *$P = 0{\cdot}0001$, before *vs.* after surgery.

revealing hypocortisolism in all the cases. Morning total serum cortisol and SAF_{23} concentrations were $37{\cdot}0 \pm 6{\cdot}0$ and $1{\cdot}7 \pm 0{\cdot}4$ nM, respectively. Levels of SAF_{23} after surgery were significantly lower than baseline ($1{\cdot}4 \pm 0{\cdot}8$ and $19{\cdot}6 \pm 15{\cdot}3$ nM, respectively; $P = 0{\cdot}0001$) (Fig. 5).

One female patient with CD (positive pituitary magnetic resonance imaging) received medical therapy with ketoconazole (600 mg/day) during 6 months. While on this drug, the patient was followed monthly with UFC and SAF_{23} measurements, normalizing UFC. On the sixth month, $SAF_{23} < 2{\cdot}1$ nM expressed the recovery of circadian rhythm of cortisol, alerting us of a possible spontaneous remission of CS. After discontinuation of ketoconazole, remission was confirmed and there is no current evidence of hypercortisolism.

Four patients with CD who underwent total hypophysectomy ($n = 2$) and selective pituitary adenomectomy ($n = 2$), relapsed 3–5 years after transsphenoidal surgery. They started on medical therapy with ketoconazole (200–800 mg/day). During the follow-up, mean UFC was < 182·0 nmol/day while SAF_{23} levels remained elevated ($10{\cdot}4 \pm 3{\cdot}5$ nM).

One patient with ectopic ACTH secretion died 3 months after thoracic exploration. Two patients with adrenal cancer who underwent unilateral adrenalectomy, normalized UFC levels ($215{\cdot}0 \pm 33{\cdot}0$ nmol/day) on mitotane. SAF_{23} could not be assessed. They died 8–10 months after surgery.

Discussion

This study demonstrates the usefulness of salivary cortisol for the diagnosis of CS. This is the first time that the reproducibility of SAF_{23} within patients with CS is described. The low intrasubject variability (11%) supports the accuracy of this methodology. A concentration of $SAF_{23} > 3{\cdot}8$ nM showed a relevant sensitivity (100%) and specificity (97·5%) for the diagnosis of CS.

In basal conditions, synchronic day collection of UFC and SAF_{23} showed a positive and significant correlation. In patients with CS, in whom salivary cortisol and serum total cortisol were simultaneously obtained, failure of normal suppression after DST was observed.

Both SAF_{dex} and F_{dex} correlated positively. A threshold value greater than 2·0 nM for SAF_{dex} and 50·0 nM for F_{dex} detected CS with 100% sensitivity and specificity. Salivary cortisol used for the assessment of the overdrive of the HPA axis as well as the derangement of normal feed-back regulation seems to be reliable and useful for the diagnosis of CS when cut-off values are clearly defined.

This salivary assay detects very low concentrations of cortisol (analytical sensitivity = 0·5 nM) with an optimal recovery (98–100%) in agreement with data reported by Raff *et al.*[21] As differences between assays may influence the optimal diagnostic criteria, in this study the same commercial RIA reagents were used to assess cortisol in the different fluids (serum, saliva and urine) gaining benefits when correlations were established.

The ICC is a measure of correlation, conformity or consistency for a data set. We orientated our study to define it for late-night salivary cortisol in patients with CS taking into account strict sampling collecting conditions and a consistent 48-h interval between samples in all the cases. Interestingly other authors, in order to diagnose CS, repeatedly assessed late-night salivary cortisol at different time intervals but reproducibility was not described.[4,22] One patient with CS showed a long-term remission of cortisol excess that could have been an intercycle phase of intermittent hypercortisolism, also called cyclic CS. The consistency of the ICC of SAF_{23} in this particular presentation of CS still needs further investigation. We found that late-night salivary cortisol measurements showed an excellent reproducibility in patients with CS with clinical features of cortisol excess as well as in healthy subjects. Our data agree with the intraindividual variability reported by Viardot *et al.*[18] in healthy subjects.

In our study, concentrations of SAF_{23} greater than 3·8 nM detected CS with 100% sensitivity and 97·5% specificity. This cut-off value is within the 97·5th percentile for healthy subjects, so only 2·5% of this population had false positive results (three cases) lying in a grey zone (4·4 – 5·2 nM). Interestingly, these three normal volunteers had not followed guidelines for saliva sampling. Subjects confessed alcohol intake ($n = 2$) and exercising ($n = 1$) before sampling. A new saliva collection on a quiet evening at home, yielded lower SAF_{23} levels to values ranging from 2·0 to 3·5 nM. The diagnostic performance of SAF_{23} found in this group is similar to data reported from other authors.[1,23]

UFC values can be extremely variable in CS, milder elevations can be found in pseudo-Cushing's states[24] but levels fourfold higher than the upper normal limit are diagnostic for CS.[24] UFC sensitivity varies from 45% to 71% (specificity 100%);[6,25] the sensitivity found in this study was 95·2% for 100% specificity (UFC cut-off value > 248·0 nmol/day). The false negative result belonged to a woman with mild CS[26] who had synchronous SAF_{23} values slightly above the cut-off level (5·2 nM). As described by others,[4,22] UFC levels in CS positively correlate with SAF_{23} concentrations.

Although the original criteria to exclude CS after DST established a value of total serum cortisol < 138·0 nM, the current accepted cut-off is < 50·0 nM enhancing sensitivity (93–96%) but lowering specificity (80%).[1] In this study we could only evaluate 17 of 21 patients with CS with DST in simultaneous saliva and serum cortisol samples. The other four patients had been previously tested in serum in other centres ($F_{dex} > 50{\cdot}0$ nM) and refused to be rechecked by us. F_{dex} and SAF_{dex} had 100% sensitivity and specificity at thresholds > 50·0 nM and > 2·0 nM, respectively. This finding agrees with Barrou *et al.*[16] who described 100% sensitivity and specificity for $SAF_{dex} > 2{\cdot}8$ nM using an in-house RIA (assay sensibility = 0·4 nM). The higher diagnostic performance of DST found in this study, in comparison with others, could be ascribed to differences in the study population and assays sensitivities.[17,26] Despite the inclusion of one patient with a mild

form of CS and another suspected to have cyclic CS, false negative results were not obtained in DST.

During the follow-up of treated patients SAF_{23} values were useful to confirm the remission or persistence of hypercortisolism.

Our experience agrees with the current concept on the accuracy and simplicity of salivary cortisol in the diagnosis of CS.

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