CLINICAL STUDY

Performance of salivary cortisol in the diagnosis of Cushing's syndrome, adrenal incidentaloma, and adrenal insufficiency

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Abstract

Objective: Salivary cortisol has recently been suggested for studies on the hypothalamic–pituitary–adrenal (HPA) axis. The lack of circadian rhythm is a marker of Cushing's syndrome (CS), and some authors have reported that low salivary cortisol levels may be a marker of adrenal insufficiency. The aim of our study was to define the role of salivary cortisol in specific diagnostic settings of HPA axis disease.

Subjects and methods: We analyzed morning salivary cortisol (MSC) and late-night salivary cortisol (LNSC) levels in 406 subjects: 52 patients with Cushing's disease (CD), 13 with ectopic CS, 17 with adrenal CS, 27 with CD in remission (a mean follow-up of 66 ± 39 months), 45 with adrenal incidentaloma, 73 assessed as having CS and then ruled out for endogenous hypercortisolism, 75 with adrenal insufficiency, and 104 healthy subjects.

Results: A LNSC value above 5.24 ng/ml differentiated CS patients from controls with high sensitivity (96.3%) and specificity (97.1%); we found higher LNSC levels in ectopic CS patients than in CD patients. We found no difference in MSC and LNSC levels between patients with CD in remission and healthy subjects. Both MSC and LNSC levels were higher in patients with adrenal incidentaloma than in healthy controls. A MSC value below 2.65 ng/ml distinguished patients with adrenal insufficiency from controls with high sensitivity (97.1%) and specificity (93.3%).

Conclusions: Salivary cortisol is a useful tool to assess endogenous cortisol excess or adrenal insufficiency and to evaluate stable CD in remission.

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Introduction

Salivary cortisol is increasingly being used to assess hyper- or hypocortisolism (1, 2). The measurement of late-night salivary cortisol (LNSC) levels in combination with the 1 mg dexamethasone suppression test (DST) and that of urinary free cortisol (UFC) levels have been proposed as the first-line laboratory tests in the diagnosis of Cushing's syndrome (CS) (3). The lack of a circadian rhythm is a peculiar marker of CS (4), so the LNSC test seems to be the best choice to screen for CS because of its noninvasive, stress-free, easy collection in outpatients (1). There is a marked variation between studies in the performance of the LNSC test, reflecting differences in laboratory assays, sample collection, severity of CS, and control groups (2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14). It is also important to have reference data not only for healthy volunteers but also for a control group of obese, hypertensive, diabetic, and depressed subjects or other clinical features of CS that

are common in the general population (3, 15). Patients with adrenal incidentaloma are another interesting control group, where mild hypothalamic–pituitary–adrenal (HPA) axis activation is found in a high percentage (16, 17).

Although the diagnosis of primary adrenal insufficiency is simple, that of the secondary form is a challenge, and dynamic tests are needed to confirm clinical suspicions (18). Healthy people show physiologically high levels of salivary cortisol when they wake up, and some authors have reported that low salivary cortisol levels may thus be a marker of adrenal insufficiency (2, 6, 19). None of these studies have looked for differences between primary and secondary adrenal insufficiency; in the latter, some residual HPA axis activity may be confounding.

The aims of our study were to determine the performance of salivary cortisol in specific diagnostic settings: i) as a first test to rule out CS; ii) in a large number of newly diagnosed CS patients of different

origin (pituitary, ectopic, and adrenal) and in patients with recurrence or remission of Cushing's disease (CD); iii) in patients with adrenal incidentaloma, and iv) in patients with adrenal insufficiency.

Materials and methods

Patients

We analyzed 406 consecutive subjects referred from January 2006 to October 2012:

- These included 82 patients with CS (age 46.6 ± 16.2 years, BMI 28 ± 5.8 kg/m²): 52 with CD, 13 with ectopic CS, and 17 with adrenal CS. CS diagnosis was made on the basis of two of three abnormal results: the lack of cortisol suppression below 50 nmol/l after the 1 mg DST, loss of cortisol circadian rhythm (considered if serum cortisol levels were above 50 nmol/l in sleeping inpatients with an indwelling venous catheter on the second night after admission or if salivary cortisol levels were higher than our local upper limit of normality of 5.24 ng/ml), and elevated UFC levels (a mean of two collections, range 30–193 nmol/l). The diagnosis of CD was made on the basis of normal or elevated serum ACTH levels, at least 80% cortisol suppression after the 8 mg DST, and response to CRH stimulation (ACTH + 50% and cortisol + 20% increase above the basal levels). Among patients with CD, 41 were newly diagnosed and 11 presented with recurrence. Ectopic CS was confirmed by bilateral petrosal inferior sinus sampling, indicating a non-pituitary ACTH source. Adrenal CS patients were characterized by low serum morning ACTH levels and radiological evidence of adrenal lesions.
- The 27 patients with CD in remission were aged 44.5 ± 14.6 years, with a mean follow-up of 66 ± 39 (from 14 to 137) months. We considered CD patients to be in remission 12 months after successful pituitary surgery, with no new clinical signs or symptoms of CS and with persistent normal HPA axis parameters based on the criteria detailed above. None were on glucocorticoid replacement therapy.
- In 45 subjects (age 57 ± 13 years, BMI 28 ± 6 kg/m²), an adrenal incidentaloma was found on CT or MRI. CS was ruled out by the criteria detailed above. In all patients, primary aldosteronism was excluded by a serum aldosterone:renin ratio < 30 and pheochromocytomas if at least two collections for urinary metanephrines and normetanephrines were normal.
- There were 73 subjects affected by common medical conditions suggestive of hypercortisolism (as summarized by Nieman *et al.* (3)), age 40 ± 15 years, BMI 33 ± 8 kg/m². Among them, 54 were obese, 12 had severe depression, 30 had metabolic syndrome, and 36 had polycystic ovary syndrome. All patients

had suppressed cortisol levels of below 50 nmol/l after the 1 mg DST and had two normal UFC consecutive collections. This non-CS group underwent clinical and biochemical follow-up for at least 12 months.

- Of the 75 patients newly diagnosed with adrenal insufficiency (age 51 ± 17 years, BMI 26 ± 4 kg/m²), 28 had the primary form (all with autoimmune adrenalitis; we excluded patients with bilateral adrenalectomy) and 47 had the secondary form (all with hypopituitarism due to neurosurgery in 27 subjects and to surgery along with radiotherapy for pituitary adenoma or craniopharyngioma in 16 patients, to pituitary apoplexy in three cases, and to traumatic brain injury and pituitary stalk interruption in one patient). Clinical diagnosis was biochemically confirmed by basal serum morning cortisol levels below 138 nmol/l in primary adrenal insufficiency and by basal cortisol levels below 138 nmol/l or cortisol response to standard- or low-dose ACTH testing below 440 nmol/l in secondary adrenal insufficiency (18). None were undergoing glucocorticoid replacement therapy or taking exogenous glucocorticoids during the study.
- The 104 healthy subjects were age matched with those in the non-CS group (age 38 ± 17 years, mean BMI 23 ± 5 kg/m²). These subjects showed no signs and symptoms of hypercortisolism and no history of severe and/or chronic illness (particularly of endocrine origin). None were taking exogenous glucocorticoids or drugs that could interfere with the HPA axis. All studies were performed in accordance with the guidelines proposed in the Declaration of Helsinki; the local ethics committee approved the protocol and all subjects gave informed consent.

Salivary sample collection and analyses

In healthy subjects and patients, morning salivary cortisol (MSC) was collected upon waking up and LNSC before going to sleep (between 1100 and 1200 h, not performed in patients with adrenal insufficiency). Patients and volunteers were advised to soak the absorbent cotton of Salivette device (Sarstedt, Numbrecht, Germany) in the saliva for 2 or 3 min; samples were then stored at +4 °C. To avoid any source of food, blood, smoke, or licorice contamination, samples were collected at least 30 min before or 2 h after taking a meal or a drink and all participants brushed their teeth after saliva collection and avoided smoking or eating licorice. After centrifugation, we obtained at least 1 ml saliva in all collections (repeating the procedures in a few days if the patient did not provide an adequate volume), and then samples were stored at –20 °C until assay with a commercially available RIA kit (Radim, Rome, Italy) with an assay sensitivity of 0.5 ng/ml, an intra-assay variation of 3%, and an inter-assay

Table 1 Morning salivary cortisol (MSC) and late-night salivary cortisol (LNSC) levels.

Population (n)	Male/female	MSC (ng/ml)	LNSC (ng/ml)
Healthy subjects (104)	42/62	7.87 ± 3.58	1.81 ± 1.41
CS (82)	22/60	24.47 ± 40.54 ^{*,‡,a,b,c}	31.94 ± 71.67 ^{*,‡,a,b,c}
CD (52)	12/40	16.23 ± 14.72 ^{*,a,c}	13.95 ± 8.83 ^{*,a,c}
Newly diagnosed CD (41)	10/31	17.46 ± 15.84 ^{§,¶}	15.15 ± 9.24 ^{§,¶}
Recurrence of CD (11)	2/9	10.11 ± 2.43	9.45 ± 5.27 ^c
CD in remission (27)	6/21	6.59 ± 4.36 [†]	1.48 ± 0.85 [†]
Ectopic CS (13)	5/8	71.28 ± 83.89 ^{*,†,‡}	126.33 ± 150.16 ^{*,†,‡}
Adrenal CS (17)	5/12	12.56 ± 8.01 [*]	14.78 ± 15.59 [*]
Non-CS (73)	19/54	10.43 ± 5.56 ^{*,}	2.25 ± 1.65
Adrenal incidentaloma (45)	16/29	10.12 ± 5.12 [*]	2.57 ± 1.46 [*]
Adrenal insufficiency (75)	37/38	1.27 ± 1.23 [*]	NC
Primary adrenal insufficiency (28)	11/17	1.40 ± 0.94	NC
Secondary adrenal insufficiency (47)	26/21	1.25 ± 1.27	NC

CD, Cushing's disease; CS, Cushing's syndrome (CD+ectopic CS+adrenal CS); NC, non-collected; ^{*}*P*<0.001 vs healthy subjects;

[†]*P*<0.01 vs CD; [‡]*P*<0.01 vs adrenal CS; [§]*P*<0.001 vs recurrence of CD; ^{||}*P*<0.01 vs CD in remission; [¶]*P*<0.05 vs newly diagnosed CD;

^a*P*<0.01 vs non-CS; ^b*P*<0.001 vs adrenal incidentaloma; ^c*P*<0.001 vs healthy subjects + non-CS.

variation of 9%. This cortisol assay does not show significant cross-reaction with cortisone, desoxycorticosterone, corticosterone, estradiol, testosterone, and 17-hydroxyprogesterone; coefficients of variation were 7% in the high range and 4% in the low range.

Statistical analysis

Continuous data are shown as mean and s.d. We first assessed the normality of distribution using the Kolmogorov–Smirnov Z test and then compared the means by Student's *t*-test for unpaired data with the correct *P* value adjusted after the Levene's test for equality of variances. We performed receiver operating curve (ROC) analyses to study the sensitivity and specificity of each threshold value. Database management and statistical analysis were performed using the SPSS 17 software package (SPSS, Inc.). Significance level was set at *P* value <5% for all tests.

Results

Concentrations of MSC and LNSC and *P* values are summarized in Table 1, and results of ROC analyses are summarized in Table 2 (to convert salivary cortisol levels from nanograms per milliliter to nanomole per liter multiply by 2.76). Considering hypercortisolism, all cutoff values for MSC had low sensitivity and specificity, so we took into account only LNSC values (depicted in Fig. 1). In contrast, for adrenal insufficiency, we took into account only MSC values.

Cushing's syndrome

LNSC levels were higher in CS patients (31.94 ± 71.67 ng/ml) than in healthy subjects (1.81 ± 1.41 ng/ml) and non-CS patients (2.57 ± 1.46 ng/ml). A threshold value of 5.24 ng/ml provided high sensitivity and specificity; the same value distinguished CS subjects

from all controls and CD patients from healthy subjects. With the proposed cutoff value, we detected 79 of 82 CS patients (51/52 CD, 15/17 adrenal CS, and 17/17 ectopic CS) and ruled out CS in the majority of non-CS patients (69 of 73). We found higher LNSC levels in patients with ectopic CS than in those with CD and adrenal CS (126.33 ± 150.16 vs 13.95 ± 8.83 vs 14.78 ± 15.59 ng/ml respectively).

Non-CS and adrenal incidentaloma

In the non-CS group, LNSC levels were similar to those in healthy subjects, whereas LNSC levels were higher in patients with adrenal incidentaloma than in healthy controls (2.57 ± 1.46 vs 1.81 ± 1.41 ng/ml), with no threshold value in terms of sensitivity or specificity. Patients with CS had higher levels of LNSC than non-CS and adrenal incidentaloma subjects (31.94 ± 71.67 vs 2.25 ± 1.65 and 2.57 ± 1.46 ng/ml respectively). We found no difference in MSC and LNSC levels between the non-CS and adrenal incidentaloma groups.

Recurrence and remission of CD

LNSC levels were higher in patients newly diagnosed with CD or recurrence of CD than in those with CD in

Table 2 Cutoff values for late-night salivary cortisol (LNSC).

Population	LNSC (ng/ml)	Sensitivity (%)	Specificity (%)	AUC
CS vs healthy subjects	5.24	96.3	97.1	0.992
CD vs healthy subjects	5.24	98.1	97.1	0.991
CS vs non-CS	5.15	96.3	94.5	0.986
CD vs non-CS	5.15	98.1	96.5	0.984
CD recurrence vs non-CS	5.15	100	95.5	0.987
CS vs adrenal incidentaloma	4.95	97.6	97.8	0.985

CD, Cushing's disease; CS, Cushing's syndrome (CD+ectopic CS+adrenal CS).

Figure 1 Plot of late-night salivary cortisol values: black line cross indicates cutoff value of 5.24 ng/ml. Salivary cortisol concentration is depicted as a logarithmic base 5 scale.

remission (15.15 ± 9.24 and 9.45 ± 5.27 vs 1.48 ± 0.85 ng/ml respectively), with no difference in LNSC levels between patients with CD in remission and healthy subjects, and higher LNSC levels in non-CS patients than in those with CD in remission (2.25 ± 1.65 vs 1.48 ± 0.85 ng/ml). We found higher LNSC levels in newly diagnosed CD patients than in those with recurrence of CD (15.15 ± 9.24 vs 9.45 ± 5.27 ng/ml). In the latter group, LNSC levels were higher than those in patients with non-CS, with a reliable threshold value.

Adrenal insufficiency

MSC levels were higher in healthy subjects than in patients with adrenal insufficiency (7.87 ± 3.58 vs 1.27 ± 1.23 ng/ml); a value of 2.65 ng/ml reliably distinguished patients with adrenal insufficiency from healthy subjects (70 of 75 patients, 97.1% sensitivity and 93.3% specificity, AUC 0.970). No difference for MSC levels was found between primary and secondary adrenal insufficiency.

Discussion

Despite improvement in laboratory and imaging techniques, diagnosis of CS remains a challenge, particularly if it is to be made before patients developed the full-blown clinical disease. In light of this, physicians may be called to exclude the different forms of CS from the most common ones in the general population: obesity, depression, hirsutism, and metabolic syndrome (3). UFC collection and the 1 mg DST are simple, but neither is able to make a certain diagnosis of CS (15, 20, 21). The LNSC test, as proposed recently (5, 6, 7, 10, 11, 12, 14), is a reliable, noninvasive, stress-free, and low-cost procedure to diagnose CS. Moreover, elevation of LNSC levels might be the first abnormality to be checked in the follow-up after surgery (22).

As reported in some studies (2, 5, 6, 7, 10, 11, 12, 14), we found that LNSC levels were higher in CS; a threshold value above 5.24 ng/ml provided a good accuracy to detect endogenous hypercortisolism, and in particular to discover CD, the most common form. LNSC was able to differentiate CS patients from the control population, which included not only healthy subjects but also patients with suspected CS, and our proposed threshold value is consistent with other data reported in the literature and obtained with RIAs (5, 11, 23).

Recently, Raff (1) has proposed a flow chart for CS diagnosis that envisages the LNSC test as the initial screening procedure, and in case of ambiguity or contradictory results, he has suggested other tests to confirm CS diagnosis. Our data are in agreement with this diagnostic plan: normal LNSC levels may rule out CS, unless there is a very high index of suspicion, particularly if a cyclic form of CS is suspected. We have undertaken a clinical and biochemical follow-up for at least 1 year to exclude any non-CS patients developing into overt CS patients, and we found none. When we retested the four cases in the non-CS group that showed slightly elevated initial levels of LNSC, we did not find the previous increase: probably the first elevated LNSC value was due to blood contamination of the salivary sample or to stress/physical exercise before their saliva sampling.

One patient with CD and two patients with adrenal CS had normal LNSC levels, but the persistent elevation of UFC levels and the lack of cortisol suppression detected by the 1 mg DST led to a correct diagnosis. Lower levels of LNSC may be due to inadequate soaking of the saliva collection device or to some fluctuation as in the case of cyclical CS. The rapid onset and progression of severe hypercorticism suggest ectopic CS, which may be difficult to pinpoint, given its being occult in up to 20% of the cases (13, 24). LNSC levels were higher in ectopic CS than in pituitary or adrenal CS, probably because higher cortisol secretion is observed in the ectopic form. We suggest that only very high levels of LNSC could induce one to suspect ectopic CS, because there is some overlap with CD when LNSC values are three to six times the proposed cutoff value of 5.24 ng/ml.

Another challenge for endocrinologists is the definition of patients that may relapse with CD after successful neurosurgery. There are data about the efficacy in relapse prediction of stimulatory tests (22, 25, 26) and fewer about salivary cortisol (27). We demonstrated higher LNSC levels in active CD patients than in those with CD in remission and also in those with non-CS than in those with CD in remission, confirming the data published by Carrasco *et al.* (27) and suggesting salivary cortisol as a simple and reliable tool to follow up patients with CD in remission. In clinical practice, some features of hypercorticism (e.g. overweight, hypertension, depression, or impaired fasting glucose) in patients with CD in remission may

only partially ameliorate, so an altered circadian cortisol rhythm may be a marker of relapse. We suggest to use the LNSC test in the follow up of CD patients after surgery, especially in patients with residual signs or symptoms that may overlap with CS features. We found higher LNSC levels in patients with newly diagnosed CD than in those with recurrence. We do not know why relapse appears with lower LNSC levels; it is probably because after neurosurgery, the patients are stringently followed up and thus diagnosis of recurrence is a prompter.

In patients with adrenal incidentaloma, LNSC levels were higher than those in healthy subjects and were effective to rule out CS: only one of 42 patients with adrenal incidentaloma showed a LNSC value higher than the proposed cutoff value, and on repeating the LNSC test a few months later, we found a normal value. Although in our series a normal LNSC value in adrenal incidentaloma can rule out CS with certainty, we did not evaluate SH due to the selection criteria of our group, so we do not suggest using the LNSC test, rather than the 1 mg DST, as a suitable screening procedure for SH, as first reported by Masserini *et al.* (28).

Diagnosis of adrenal insufficiency is a matter of clinical debate, although a recent meta-analysis has suggested using serum cortisol after ACTH injection (18), but apart from dynamic testing, the use of salivary cortisol is less established in adrenal insufficiency, because both Deutschbein *et al.* (2) and Restituto *et al.* (6) have reposed a low accuracy for diagnosis. In our series, we confirmed our previous results that MSC levels are lower in patients with established adrenal insufficiency than in healthy controls (19). We still use dynamic tests to discover adrenal insufficiency, but we suggest using periodic salivary cortisol evaluation to follow up patients (especially with pituitary disease) in order to perform the dynamic test at the correct time.

Our study has several limitations. It is noteworthy that methodologies of salivary cortisol analyses are critical due to assay differences and control populations considered, so we suggest that every endocrinologist use cutoff values of known sensitivity and specificity based on the local measurements at various times of the day and based on a control population with obesity, diabetes, or depression, as has been suggested recently (24).

Our experience is in agreement with the concept of the accuracy and feasibility of LNSC as a first step in the diagnostic work-up of the HPA axis disorder (both CS and adrenal insufficiency). We suggest that periodic LNSC measurement may represent a simple and effective way to follow up CD patients in remission and for early identification of patients that relapse.

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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