Usefulness of salivary cortisol in the diagnosis of hypercortisolism: comparison with serum and urinary cortisol

Luca Manetti, Giuseppe Rossi, Lucia Grasso, Valentina Raffaelli, Ilaria Scattina, Simone Del Sarto, Mirco Cosottini, Aldo Iannelli, Maurizio Gasperi, Fausto Bogazzi and Enio Martino

Abstract

Objective: Several tests have been proposed to diagnose patients with Cushing’s syndrome (CS). The aims of the study were: i) to evaluate the performance of salivary cortisol (SC) in hypercortisolism and ii) to compare SC with serum cortisol (SeC) and urinary cortisol.

Design and patients: This was a diagnostic study. Twenty-seven patients with untreated Cushing’s disease (CD untr), 21 women consuming oral contraceptive pill (OCP), 18 pregnant women, and 89 healthy subjects (controls) were enrolled.

Methods: SC and SeC at baseline and after the low-dose dexamethasone suppression test (LDDST) and urinary free cortisol (UFC) were measured.

Results: Midnight SC had a sensitivity of 100% in the CD untr group and a specificity of 97.7% in the controls. Specificity remained high (95.2%) in women taking OCP, while in pregnant women, it decreased to 83.3%. SC after the LDDST showed a sensitivity of 96.3% in the CD untr group; specificity was 97.7% in the controls and 90.5% in OCP women. Midnight SeC had a sensitivity of 100% in the CD untr group. SeC after the LDDST had a sensitivity of 100% in the CD untr group while specificity was 92.6% in the CD untr group while specificity was 97.7% in the controls and 100% in the OCP group.

Conclusions: SC is a reliable parameter for the diagnosis of severe hypercortisolism, with high sensitivity and specificity. In women during pregnancy or taking OCP, the measurement of SC, identifying the free fraction, could be helpful to exclude CS.

Introduction

The diagnosis of Cushing’s syndrome (CS) remains a challenge in clinical endocrinology. Thus, several diagnostic tests are widely used for the screening and diagnosis of endogenous cortisol excess. Currently, available biochemical screening tests for the diagnosis of CS have limitations: assessment of diurnal rhythm and measurement of unstressed late-night serum cortisol (SeC) concentrations require hospitalization and, for midnight SeC (MSeC), test accuracy was underestimated (1, 2, 3, 4). Urinary free cortisol (UFC) provides an integrated assessment of cortisol secretion over a 24-h period; however, urine measurements may be inaccurate because of improper collection technique (3). Once an accurate urine collection is achieved, the sensitivity of UFC measurement is 45–71% when specificity is set at 100% (5). The 1 mg overnight dexamethasone suppression test (DST) and the 2 mg dexamethasone 2-day suppression test (low-dose DST (LDDST)) are simple and inexpensive; failure of normal suppression of SeC is a well-known biochemical screening test when CS is suspected, showing a sensitivity and specificity >95 and 80% respectively (3, 6, 7).

Salivary cortisol (SC) has recently been used by many centers as a first-line diagnostic test for CS (8, 9, 10, 11). SC reflects the free fraction of total SeC representing the unbound, biologically active form of SeC and is not influenced by alterations in binding protein (11). Therefore, it could be a safe and practical alternative, being a noninvasive stress-free procedure, easier to collect, even at home, saving the costs of hospitalization, but its accuracy is still debated.

Pregnancy represents a unique biological state because of the known physiological increase in total and free SeC levels (12, 13, 14), while it is known that estrogens administered alone or in combination with progesterin in the oral contraceptive pill (OCP) increase cortisol-binding globulin (CBG) and total SeC concentrations (15). Few data are available in the literature on the determination of SC in pregnancy or OCP therapy.
The aims of the study were: i) to analyze sensitivity and specificity of SC in patients with untreated Cushing’s disease (CD untr) and in conditions of altered CBG concentrations such as pregnancy and the use of OCP; and ii) to compare the performance of SC with SeC and urinary cortisol.

Materials and methods

Study design

This was a diagnostic study assessing SC in healthy subjects, patients with CD untr, women consuming OCP, and women during pregnancy conducted at the Department of Endocrinology, University of Pisa, in the period from January 2009 to December 2011. The study was approved by the Ethical Board of the Department of Endocrinology on a previously obtained informed consent.

At baseline, for CS diagnosis, the study protocol included an accurate clinical history and physical examination. The hormonal evaluation included a salivary and blood sample for cortisol measurement obtained at 0800, 1600, and 2400 h, plasma ACTH at 0800 h, and UFC (collected over 24 h). Patients with CD were hospitalized, while in the other groups, the collection of SC, SeC, and UFC occurred in an outpatient setting. Owing to this setting, MSeC in the controls and women during pregnancy conducted at the Department of Endocrinology on a previously obtained informed consent.

The determinations of SC were not taken into account for the requirements for a correct execution of the test were not guaranteed. The corticotropin-releasing hormone (CRH) test was performed after an i.v. administration of 100 µg ovine CRH. Dexamethasone at a dose of 0.5 mg for the LDDST was administered orally strictly every 6 h for 48 h (LDDST), and at a dose of 8 mg for the high-dose DST administered at 2400 h. Blood and salivary samples were collected for serum and SC measurement at 0800 h after 48 h following the first dose of dexamethasone for the LDDST and at 0800 h the next morning following a high dose of dexamethasone. Magnetic resonance imaging (MRI), before and after the administration of gadolinium, was performed in all CD patients. An inferior petrosal sinus sampling after the CRH stimulation test was performed when required. Patients with CD underwent transphenoidal pituitary surgery; a confirmed diagnosis of CD was made in all patients on the basis of the demonstration of pituitary corticotroph adenoma at pathological examination.

The main clinical features of the study groups are shown in Table 1.

Table 1 Clinical features of the study population.

<table>
<thead>
<tr>
<th></th>
<th>CD untr (n=27)</th>
<th>OCP (n=21)</th>
<th>Pregnancy (n=18)</th>
<th>Controls (n=89)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex (F/M)</strong>*</td>
<td>24/3</td>
<td>21</td>
<td>18</td>
<td>52/37</td>
</tr>
<tr>
<td><strong>Age (years)</strong>*</td>
<td>(16–80)</td>
<td>(26–45)</td>
<td>(26–43)</td>
<td>(20–80)</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>31.4±8.1</td>
<td>20.6±1.85</td>
<td>ND</td>
<td>24.0±3.8</td>
</tr>
<tr>
<td></td>
<td>(21–63)</td>
<td>(17–24)</td>
<td></td>
<td>(17–36)</td>
</tr>
</tbody>
</table>

CD untr, patients with active Cushing’s disease; OCP, women consuming oral contraceptive pill.

No patient in the study, at the time of the evaluation, was taking metyrapone, prednisolone, prednisone, or other synthetic steroids.

The study population was composed of a group of patients with CD untr and three control groups, as described below:

- Twenty-seven patients (24 women, three men) with CD untr were enrolled in the study. Diagnosis of CD was based on: i) clinical features of hypercortisolism; ii) absence of a circadian rhythm of SeC; iii) inappropriately high morning plasma ACTH concentrations (> 20 pg/ml); iv) failure of SeC suppression (≤ 18 ng/ml) after the LDDST; v) MRI confirmation of a pituitary micro- or macroadenoma; and vi) inferior petrosal sinus gradient > 3 after CRH stimulation when appropriate (3). Twenty-two patients were affected by a microadenoma, detectable on MRI in 15 patients, while the remaining had a pituitary macroadenoma. Patients with CD underwent transphenoidal pituitary surgery. A confirmed diagnosis of CD was made in all patients on the basis of the demonstration of pituitary corticotroph adenoma at pathological examination.

- The first control group included 89 consecutive healthy subjects (52 women, 37 men). No control subject was taking drugs interfering with the assay of SeC, SC, and urinary cortisol.

- Twenty-one women consuming OCP and 18 women during the second and the third trimester of pregnancy were enrolled in the study as control groups. None of the women after the withdrawal of OCP therapy, as well as none of the pregnant women after delivery, showed clinical or biochemical signs of hypercortisolism (data not shown).


**SC collection and assay**

Saliva was collected with a commercially available device (Salivette Tube System; Sarstedt, Nümbrecht, Germany). The subjects had to remove the swab and gently chew for 2 min. The collection of the sample had to be at least 1 h after taking food or drink and having brushed their teeth (16). The swab was then placed in a container inside the tube. Salivary samples were centrifuged at room temperature for 10 min and stored at −20 °C until assayed. For the determination of SC, a RIA kit (Immunootech, Marseille, France) was used. The analytical and functional sensitivity of the method was 0.3 and 0.5 ng/ml respectively. Based on our data, the intra-assay coefficient of variation (CV) was < 6.0% and the inter-assay CV was < 6.2%. In order to determine whether saliva samples could be stored at room temperature, we took 20 samples of SC which were kept at room temperature for a period of 7 days. The measurement of SC carried out after storage at room temperature was comparable with that performed pre-storage \( (P = 0.8) \). The cross-reactivity of the method with dexamethasone was < 0.5% and therefore not significant (17, 18, 19, 20).

**Hormone assays**

SeC (Immunootech), plasma ACTH (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA), and UFC (DSL-2100 Active, Cortisol RIA, Webster, TX, USA) were assayed by commercial kits. Normal values in our laboratory were as follows: early morning cortisol, 85–260 ng/ml; early morning ACTH, 9–52 pg/ml; UFC, 55–346 μg/24 h.

**Statistical analysis**

Data are expressed as mean ± S.D. for quantitative variables and as a percentage for qualitative variables. For continuous variables, the difference between the control group and each of the other groups was evaluated by the ANOVA test and the nonparametric Wilcoxon’s test for independent data. In the presence of heteroscedasticity, the Welch ANOVA test was used.

The diagnostic tests were compared by ROC curves. The area under the receiver operating characteristic (ROC) curve was computed by the nonparametric method while the standard error by the DeLong, DeLong, and Clarke-Pearson method. The comparison between two ROC curves was performed by the \( \chi^2 \) test.

The reference limits of SC, SeC, and urinary cortisol, based on the healthy subjects, were computed using a nonparametric method: 97.5th percentile of the data distribution. A SC, SeC, and urinary cortisol value higher than the corresponding reference limit indicated hypercortisolism.

The validity of the reference limit for each test was evaluated by considering its ability to correctly classify control subjects and patients with CD untr. The performance indices used were sensitivity for the CD untr group and specificity for the control groups. The 95% confidence interval of sensitivity and specificity was computed by the Clopper–Pearson method. The comparison between different diagnostic tests for sensitivity and specificity was performed by Fisher’s exact test. The correlation between SeC after the LDDST (SC\textsubscript{LDDST}) and SeC after the LDDST (SeC\textsubscript{LDDST}) was evaluated by the Pearson correlation.

All statistical tests were two-tailed; a \( P \) value < 0.05 was considered to be statistically significant. Statistical analysis was performed by STATA Software, version 10.0 (STATACorp., College Station, TX, USA), and by StatXact Software, version 4 (Cytel Software Corporation, Cambridge, MA, USA).

**Results**

Midnight SC (MSC) and SC\textsubscript{LDDST} were not influenced by age, sex, and BMI, both in the controls and in the CD untr group. Therefore, reference limits for SC were computed regardless of age, sex, and BMI. The upper limits were \( \leq 2.77 \) and \( \leq 1.22 \) ng/ml for MSC and SC\textsubscript{LDDST} respectively, using the 97.5th percentile of the data distribution in healthy subjects. For MSeC, SeC\textsubscript{LDDST} and UFC, the reference limits were \( \leq 18 \), \( \leq 18 \) ng/ml, and \( \leq 346 \) μg/24 h respectively.

**Untreated CD group (CD untr)**

Data are shown in Table 2. In CD untr patients, SC concentrations were significantly higher than the controls throughout the day \( (P < 0.0001) \) and after the LDDST \( (P < 0.0001) \). SeC levels were significantly higher than those of the controls in all the determinations performed \( (0800 \) and \( 1600 \) h, \( P < 0.0001) \) and after the DST \( (P < 0.0001) \). UFC was significantly higher in patients with active disease \( (P < 0.0001) \) compared with the controls.

**Women consuming OCP**

In women receiving OCP, SC was not statistically different than the controls throughout the day, except for the determination after the LDDST \( (P = 0.006) \). In this group, there was a clear elevation of SeC concentration, as expected, compared with the controls \( (0800 \) h, \( P < 0.0001) \) and \( 1600 \) h, \( P = 0.0004) \) and even after the LDDST \( (P < 0.0001) \). Finally, the UFC concentrations were similar in women assuming OCP therapy and in controls (Table 2).

**Pregnant women (pregnancy)**

In pregnant women, MSC was significantly higher than the controls \( (P < 0.0001) \), while in the other two determinations, it was comparable with healthy control subjects.
Table 2 Test results (mean ± s.d. and range) of the study population.

<table>
<thead>
<tr>
<th></th>
<th>CD untr (n=27)</th>
<th>OCP (n=21)</th>
<th>Pregnancy (n=18)</th>
<th>Controls (n=89)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC0800 h (ng/ml)</td>
<td>14.0±9.29</td>
<td>&lt;0.0001</td>
<td>8.27±3.71</td>
<td>0.9</td>
</tr>
<tr>
<td>SC1600 h (ng/ml)</td>
<td>11.0±6.96</td>
<td>2.81±0.98</td>
<td>3.49±1.85</td>
<td>0.5</td>
</tr>
<tr>
<td>MSC (ng/ml)</td>
<td>10.8±9.10</td>
<td>1.51±0.70</td>
<td>2.41±1.33</td>
<td>0.3</td>
</tr>
<tr>
<td>SC1600 h (ng/ml)</td>
<td>2.83–29.6</td>
<td>1.43–5.96</td>
<td>1.60–9.13</td>
<td>0.68–8.80</td>
</tr>
<tr>
<td>UFC (g/24 h)</td>
<td>3.88–49.1</td>
<td>0.70–3.74</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SC1600 h (ng/ml)</td>
<td>6.39±6.19</td>
<td>0.74±0.39</td>
<td>0.006</td>
<td>ND</td>
</tr>
<tr>
<td>SeC0800 h (ng/ml)</td>
<td>211±85.7</td>
<td>211±54.8</td>
<td>&lt;0.0001</td>
<td>ND</td>
</tr>
<tr>
<td>SeC1600 h (ng/ml)</td>
<td>112–415</td>
<td>119–322</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MSeC (ng/ml)</td>
<td>161±72.3</td>
<td>16–200</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SeCLDDST (ng/ml)</td>
<td>57–324</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>UFC (µg/24 h)</td>
<td>926±1100</td>
<td>161±70.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>301–5924</td>
<td>80–345</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

CD untr, patients with active Cushings’ disease; OCP, women consuming oral contraceptive pill; SC0800 h, early morning salivary cortisol; SC1600 h, afternoon salivary cortisol; MSC, midnight salivary cortisol; SC1600 h, midnight salivary cortisol; SeC0800 h, early morning serum cortisol; SeCLDDST, serum cortisol after the low-dose dexamethasone suppression test; SeC1600 h, afternoon serum cortisol; MSeC, midnight serum cortisol; SeCLDDST, serum cortisol after the low-dose dexamethasone suppression test; UFC, urinary free cortisol; ND, not done. The P values express the comparison between the group and the control population.

Correlations between SC and SeC

SC1600 h was positively correlated with SeCLDDST in the CD untr (r=0.93, P<0.0001), OCP (r=0.55, P=0.009) and control groups (r=0.63, P<0.0001) (Fig. 1). MSC was positively correlated with MSeC in the CD untr group (r=0.80, P<0.0001).

Diagnostic performance of SC in the study groups

Midnight SC At a cutoff level of 18 ng/ml, MSeC had a sensitivity of 100% to identify patients with CD untr.

SeC after the LDDST Data are summarized in Table 3. Using the SeC cutoff of 18 ng/ml after the DST, sensitivity was 100% in patients with CD untr while specificity was 97.7% in the controls. Specificity in women taking OCP was 61.9% and in fact eight subjects had cortisol values above the threshold after LDDST.

Diagnostic performance of UFC in the study groups

Using the threshold of 346 µg/24 h for UFC, sensitivity was 92.6% in the CD untr group while specificity was 97.7% in the controls (Table 3). Specificity was 100% in the OCP group.

Comparison between the tests

The comparison of ROC curves for the CD untr and control groups did not show any significant difference between the tests. The comparison between the tests for sensitivity and specificity showed a significant or near-to-significant difference as follows: MSC vs SeCLDDST (P=0.020), SCCLDDST vs SeCLDDST (P=0.067), and SeCLDDST vs UFC (P=0.003) in OCP (Table 3).
Discussion

In the present study, we studied a group of patients with a confirmed diagnosis of CD. In a recent review, Raff reported that measurement of an elevated late-night SC has a >90% sensitivity and specificity for the diagnosis of CS (11), while a recently published meta-analysis on SC measurement in CS took into account only seven studies that contained sufficient information to be processed (10). A total of 339 patients with CS revealed for SC a pooled sensitivity of 92% and a specificity of 96% (5, 21, 22, 23, 24, 25, 26). In our series, a cutoff of 2.77 ng/ml for MSC obtained a sensitivity of 100% to identify patients with CD untr and a specificity of 97.7% in the controls, the results being similar to those described in the literature (5, 7, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31). More limited data are available regarding the determination of SC after the DST and not always as reliable as MSC (22, 28, 31, 32, 33). In our series of CD untr patients, SC LDDST obtained an excellent performance: using the threshold of 1.22 ng/ml, we obtained a sensitivity of 96% with a specificity of 97.7% in the controls.

When we analyzed the other tests performed on patients with CD untr, we observed that MSeC had a high sensitivity (100%) as well as SeC LDDST (100%), while the sensitivity of urinary cortisol was slightly reduced (92.6%). In summary, both MSC and SC LDDST are good parameters for evaluating patients with severe hypercortisolism, while urinary cortisol has a lower sensitivity (92.6%).

It has long been known that estrogen administration in the OCP increases CBG and total plasma cortisol concentrations (34, 35). In this study, the mean values of SC in women consuming OCP were similar to those of controls throughout the day, confirming that SC, as an unbound fraction, is not affected by the modification of CBG. In fact, the specificity of MSC was 95.2%. After the LDDST, SC was significantly higher than that in the controls, although well below the cutoff set to 1.22 ng/ml. With this threshold, specificity was 90.5%; therefore, only two women on estrogen therapy had no suppressed cortisol values, suggesting good reliability of the test. On the contrary, and as reported in the literature, the performance of total SeC is very low, due to the influence of CBG, with a specificity of SeCLDDST of 61.9%. It would therefore be useful in such patients to establish a specific cutoff for SeCLDDST, which in our case might be >25 ng/ml, allowing to identify 20 out of 21 patients. UFC was comparable with the controls with an excellent diagnostic performance (100%).

An increase in free cortisol fraction was observed in pregnancy, maybe due to the alterations in the regulation of the maternal hypothalamic–pituitary–adrenal axis. In this regard, there are several hypothesis: i) autonomous and not subject to normal feedback control CRH and ACTH production by the placenta; ii) increased responsiveness of the adrenal glands to ACTH during pregnancy; and iii) state of refractoriness to cortisol action during pregnancy, resulting in resetting of the HPA axis (13, 15, 36, 37). Our data showed that during pregnancy, MSC was increased compared with the controls (P<0.0001), but the

![Figure 1](image_url)

**Figure 1** Correlation between SC LDDST vs SeC LDDST in patients with active Cushing's disease (CD untr), women consuming estrogen therapy (OCP), and controls. SC LDDST, salivary cortisol after the dexamethasone suppression test; SeC LDDST, serum cortisol after the dexamethasone suppression test.
average is much lower than in patients with overt hypercortisolism (2.41 vs 10.80 ng/ml). The mean values during the day were kept within the limits and were comparable with respect to the control population. Using a cutoff of 2.77 ng/ml, the specificity of MSC was 83.3%, so three women were identified as hypercortisolemic. This observation seems to align with the data reported in the literature: the free fraction of cortisol during pregnancy may be affected, although the mean values were slightly higher than the controls and significantly lower than a patient with severe hypercortisolism. Also, in this group of subjects, it would be useful to establish a specific cutoff for MSC: a threshold of 3.9–4.0 ng/ml would allow to identify a single subject as hypercortisolemic.

The main limitation in the use of SC measured by RIA or other immunoassay method is cross-reactivity by other steroids, exogenous glucocorticoids, and endogenous cortisol precursor and metabolites (17, 18, 19, 20). This is partly responsible for the variable cutoffs for MSC reported by various authors, so that each laboratory should identify their normal range in a large series of subjects. However, immunoassay methods are cheaper and available in many laboratories. The main advantage of SC measurement is an easy collection and an easy maintenance of the samples by avoiding hospitalization of the patient.

In conclusion, our study confirms that SC is a reliable parameter for the diagnosis of severe hypercortisolism, with a high sensitivity and specificity of MSC and SeCLDDST. In women during pregnancy or OCP therapy, the investigation of possible cortisol excess and the differentiation between CS and physiological hypercortisolism could be more difficult due to the increase in the total fraction of cortisol: the measurement of SC, identifying the free fraction, could be helpful in differentiating these two conditions.

### 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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Salivary cortisol in hypercortisolism

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