The limited role of midnight salivary cortisol levels in the diagnosis of subclinical hypercortisolism in patients with adrenal incidentaloma

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Abstract

Objective: The criteria for defining subclinical hypercortisolism (SH) are debated and a real gold standard test or combination of tests is lacking. Recently, late-night salivary cortisol (MSC) has been described as a sensitive and easy-to-perform marker for diagnosing overt hypercortisolism. No data are available on the role of MSC in the diagnosis of SH. The aim of this study was to evaluate the sensitivity and specificity of MSC levels in the diagnosis of SH in patients with adrenal incidentalomas (AI).

Methods: In 103 (females/males, 69/34) patients with AI, MSC levels were studied. One milligram overnight dexamethasone suppression test (DST), urinary-free cortisol (UFC), and ACTH plasma levels were also evaluated. Patients were defined as affected by SH if they showed two of the following criteria:

- DST: \[ < 83 \text{ nmol/l} \]
- ACTH: \[ < 2.2 \text{ pmol/l} \]
- UFC: \[ > 193 \text{ nmol/24 h} \]

Results: No difference in MSC levels in patients with SH (3.1 ± 3.1 nmol/l) compared with patients without SH (2.2 ± 2.8 nmol/l) was observed. In patients with SH, MSC levels were significantly correlated with DST (\[ r = 0.4, P < 0.05 \]). Using the cut-off of 5.1 nmol/l, the sensitivity and specificity of MSC levels for diagnosis of SH is 22.7 and 87.7% respectively.

Conclusion: In patients with AI, normal levels of MSC do not exclude SH, whereas high levels may suggest the presence of SH identified by conventional tests. Thus, MSC is not suitable as a screening test, although it may be used in conjunction with other tests as the confirming test in selected patients.

Introduction

In the last years, the increased application of abdominal imaging led to the frequent finding of incidentally detected adrenal masses, adrenal incidentalomas (AI). As a consequence, AI have become a common clinical problem, and their prevalence ranges between 0.3 and 4.4% (1, 2). The definition of incidentaloma includes a wide spectrum of pathological entities, among which the most frequent is adenoma. By definition, patients with AI do not show any sign or symptom of overt hypercortisolism (Cushing’s syndrome, CS). However, these masses may secrete cortisol autonomously in 5–20% of cases determining a condition known as ‘subclinical hypercortisolism’ (SH) or ‘subclinical CS’ (3–10). This condition has been described to be associated with high prevalence of hypertension, diabetes mellitus, obesity, dyslipidemia, and osteoporosis (9, 11–15). Unfortunately, the diagnosis of this entity is difficult due to the lack of specific symptoms and of a gold standard endocrine test. Indeed, in order to ascertain the presence of subtle cortisol excess, the same endocrine tests used for the diagnosis of overt hypercortisolism are employed. Nowadays, at least two criteria among reduced cortisol suppression after 1 mg overnight dexamethasone suppression test (DST), low morning ACTH levels, high 24 h urinary-free cortisol (UFC), and high midnight serum cortisol levels (3) are required to make diagnosis of SH. However, the cut-off of these tests, as well as the sensitivity and specificity of their combination in diagnosing SH, is still debated, since the lack of clinical manifestation makes it difficult to distinguish between true and false-positive results. Moreover, some of these tests are cumbersome and expensive.

Recently, the determination of salivary cortisol at bedtime (2300 h) has been described as a sensitive and easy-to-perform marker for the diagnosis of CS, and some authors have proposed the determination of bedtime salivary cortisol in the place of serum cortisol at midnight (16–21). The aim of this study is to evaluate
the possible utility of determination of bedtime salivary cortisol in the diagnosis of SH in patients with AI.

**Subjects and methods**

**Subjects**

A total of 103 (69 females, 34 males) consecutive patients with unilateral AI were recruited. The inclusion criteria were the following: absence of depression and alcoholism, no administration of drugs influencing cortisol and dexamethasone metabolism or cortisol secretion, no signs or symptoms of cortisol excess including moon faces, striae rubrae, skin atrophy or buffalo hump, and no evidence of metastatic disease.

All incidentalomas were discovered by abdominal ultrasound or CT scan, performed for the evaluation of unrelated diseases (such as renal or biliary colic, non-specific abdominal pain, or during a normal check-up). The findings of incidentaloma by ultrasound were confirmed with CT scan.

Pheochromocytoma and aldosteronoma were excluded by appropriate endocrine measurements (24-hour urinary catecholamines and plasma renin activity and aldosterone in the recumbent position and after 1 h of upright position).

Written informed consent was obtained by all subjects and the study was approved by the local ethics committees.

In all patients, clinical examination was performed for the presence of hypertension, dyslipidemia, and diabetes mellitus. Subjects with systolic blood pressure of $\geq 130$ mmHg and/or diastolic blood pressure $\geq 85$ mmHg and/or on antihypertensive treatment were defined as hypertensive. Dyslipidemia was defined as serum triglyceride levels of at least 1.69 mmol/l (150 mg/dl) or high-density lipoprotein cholesterol levels of less than 0.70 mmol/l (40 mg/dl) and men and 1.29 mmol/l (50 mg/dl) in women (22). Patients were also considered dyslipidemic if any anti-dyslipidemic treatment was given. Diabetes mellitus was diagnosed using World Health Organization (WHO) criteria (23) and patients were also considered diabetic if any hypoglycemic drugs were given.

In all patients, the following determinations were also performed: 24-hour UFC (normal values: 27.6–193 nmol/24 h), plasma ACTH at 0800 h (normal values: 0–4.4 pmol/l), salivary cortisol levels at 0800 h (mSC: normal values: 4.9–32.1 nmol/l); after UFC, ACTH, and salivary cortisol determination, serum cortisol levels were measured at 0800 h after 1 mg overnight DST and salivary cortisol levels were evaluated at 2300 h (midnight salivary cortisol, MSC; normal values: 0.68–5.1 nmol/l). The upper limit of MSC corresponds to the 95th percentile value of our reference population. In order to confirm the autonomous cortisol secretion, in patients with SH and ACTH levels higher than 1.1 pmol/l, $^{131}$I-6$\beta$-iodomethyl-norcholesterol scintigraphy was performed. In the presence of ACTH levels higher than 2.2 pmol/l, CRH test was also performed in order to exclude pituitary hypercortisolism.

SH was diagnosed if the subjects had at least two of the following criteria: incomplete suppression of cortisol after 1 mg overnight DST (DST > 83 nmol/l), UFC level higher than 193 nmol/24 h, and morning ACTH levels lower than 2.2 pmol/l. According to the presence of SH, the patients were subdivided in two groups: 22 subjects with SH (SH+) and 81 without SH (SH-).

**Methods**

Clinical examinations included weight and height with calculation of body mass index (BMI) and systolic and diastolic blood pressure measured according to the WHO International Society of Hypertension Guidelines.

Saliva was collected by chewing a cylindrical cotton swab (Salivette, Sarstedt, Nümbrecht, Germany) for about 2 min at 2300 h at home and at 0800 h. At least 3 h before the collection, the subjects were told not to eat and brush their teeth. The specimens, collected at home, were refrigerated at a temperature of 2–8°C and were brought to the laboratory within 1 day. The received samples were centrifuged at 3000 r.p.m. for 3 min. The cotton swab was removed, and the collected saliva samples were frozen at $-20$ °C until assayed. Serum samples were collected at 0800 h.

Salivary cortisol levels were measured immunofluorimetrically (AutoDELFIA; Wallac Inc., OY, Turku, Finland) (24, 25). Intra- and interassay coefficients of variations were 6.2 and 3.7% respectively.

Serum cortisol and UFC levels were determined immunofluorimetrically by AutoDELFIA kit (Wallac Inc.; serum ACTH levels (mean of three determinations at 20-min intervals) were measured by chemoluminescent immunometric assay (Immulite 2000, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA).

Glucose, total cholesterol, high-density cholesterol, and triglycerides were measured by standard procedures.

**Statistical analysis**

Statistical analyses were performed using SPSS software, version 12.0 (SPSS, Chicago, IL, USA).

The results are expressed as mean $\pm$ S.D. Comparison of continuous variables among groups was done using Student’s $t$-test. Categorical variables were compared by $\chi^2$ test.

Bivariate correlations between variables were tested using Pearson correlation test. Logistic regression analysis assessed the relationship between the presence of diabetes, hypertension, and dyslipidemia taken separately with ACTH or UFC, or DST or MSC, after adjustment for age and BMI. Values of $P < 0.05$ were considered significant.
Results

Clinical characteristics of the subjects included in the two groups are reported in Table 1.

The two groups were comparable for age, gender, BMI, prevalence of hypertension, dyslipidemia, and diabetes mellitus. Patients with SH when compared with patients without SH showed larger adrenal mass (Table 1).

Patients with SH showed higher UFC levels and serum cortisol levels after DST and lower ACTH levels, when compared with patients without SH (Table 2). However, SH+ patients did not show differences in MSC and moSC levels when compared with SH− (Table 2; Fig. 1).

In SH+ patients, DST was > 83 nmol/l in 86.4%, ACTH < 2.2 pmol/l in 86.4%, and UFC > 193 nmol/24 h in 31.8% of cases. In SH− patients, DST was < 83 nmol/l in 96.3%, ACTH > 2.2 pmol/l in 59.3%, and UFC < 193 nmol/24 h in 92.6% of cases.

Considering all our patients with AI, DST was negatively correlated with ACTH levels (P < 0.05, r = −0.2) and directly with UFC (P < 0.05, r = 0.2). MSC did not correlate with other parameters of hypothalamic–pituitary–adrenal (HPA) axis (UFC, DST, and ACTH levels).

When the two groups were analyzed separately, in SH+ patients MSC levels were directly correlated with DST (P < 0.05 r = 0.4) (Fig. 2), and tended to be correlated with UFC (P = 0.06 r = 0.4). No correlation between MSC levels and other parameters of HPA axis activity and clinical parameters was found. Conversely, in SH− group, MSC levels did not correlate with any parameter of HPA axis function.

No parameter of HPA axis function was correlated with the presence of diabetes mellitus, dyslipidemia, and hypertension taken separately after adjustment for age and BMI as shown by logistic regression analysis.

Table 1 Clinical characteristics of patients with and without subclinical hypercortisolism.

<table>
<thead>
<tr>
<th></th>
<th>Patients with SH (n=22)</th>
<th>Patients without SH (n=81)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.2 ± 8.7 (44–77)</td>
<td>63.6 ± 9.3 (27–82)</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>14/8</td>
<td>55/26</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.5 ± 4.6 (21–36)</td>
<td>27.8 ± 4.2 (20–39)</td>
</tr>
<tr>
<td>Diameter of adrenal mass (cm)</td>
<td>2.9 ± 1.2*</td>
<td>2.2 ± 0.9</td>
</tr>
<tr>
<td>Patients with hypertension (n)</td>
<td>11 (50)</td>
<td>47 (58)</td>
</tr>
<tr>
<td>Patients with dyslipidemia (n)</td>
<td>5 (23)</td>
<td>22 (27)</td>
</tr>
<tr>
<td>Patients with diabetes mellitus (n)</td>
<td>6 (27)</td>
<td>13 (16)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.D. with range and percentages in parentheses. *P<0.05 versus patients without SH; BMI, body mass index; SH, subclinical hypercortisolism; SH was diagnosed if two of the following criteria were present: cortisol after 1 mg overnight dexamethasone suppression test > 83 nmol/l, 24-hour urinary-free cortisol greater than 193 nmol/24 h, and morning ACTH levels < 2.2 pmol/l.

Table 2 Parameters of hypothalamic–pituitary–adrenal (HPA) axis activity in patients with and without subclinical hypercortisolism.

<table>
<thead>
<tr>
<th></th>
<th>Patients with SH (n=22)</th>
<th>Patients without SH (n=81)</th>
</tr>
</thead>
<tbody>
<tr>
<td>moSC (nmol/l)</td>
<td>15.3 ± 8.6</td>
<td>12.9 ± 6</td>
</tr>
<tr>
<td>MSC (nmol/l)</td>
<td>3.1 ± 3.1</td>
<td>2.2 ± 2.8</td>
</tr>
<tr>
<td>DST (nmol/l)</td>
<td>126.9 ± 69*</td>
<td>52.4 ± 30.4</td>
</tr>
<tr>
<td>ACTH (pmol/l)</td>
<td>1.7 ± 0.9†</td>
<td>3 ± 1.9</td>
</tr>
<tr>
<td>UFC (nmol/24 h)</td>
<td>157.0 ± 97 6*</td>
<td>100.4 ± 59.9</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.D. *P<0.0001 versus patients without SH; †P=0.002 versus patients without SH; ‡P=0.001 versus patients without SH; SH, subclinical hypercortisolism; MSC, salivary cortisol at 2300 h; moSC, salivary cortisol at 0800 h; DST, cortisol after 1 mg overnight dexamethasone suppression test; UFC, 24-hour urinary-free cortisol. SH was diagnosed if two of the following criteria were present: DST > 83 nmol/l, UFC > 193 nmol/24 h, and ACTH < 2.2 pmol/l.

Using the upper cut-off of 5.1 nmol/l, which corresponds to the 95th percentile of our reference population, the sensitivity and specificity of MSC levels for the diagnosis of SH was 22.7 and 87.7% respectively (Table 3).

Since the cut-off of cortisol after DST is still a matter of debate in diagnosing SH, we repeated the analyses using a DST cut-off of 50 nmol/l. The results about the differences between SH+ and SH− patients in the parameters of HPA axis function, prevalence of hypertension, dyslipidemia, and diabetes mellitus, and about the sensitivity and specificity of MSC were comparable with those obtained with the DST cut-off of 83 nmol/l (data not shown).

Figure 1 Midnight salivary cortisol levels in patients with and without subclinical hypercortisolism.

Discussion

This is the first study that evaluates the possible use of salivary cortisol for diagnosing SH in patients with AI.

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Salivary cortisol offers several advantages: it reflects the unbound fraction of circulating cortisol, it is not influenced by saliva flow rate, and it can be collected at home, eliminating the cost of hospitalization and the stress of hospital visit (26).

In the present study, we evaluated the possible role of MSC in the diagnosis of SH in patients with AI. We found that MSC levels were not different between patients with and without SH and that MSC directly correlated with DST in patients with SH. A sensitivity and specificity of MSC of 22.7 and 87.7% respectively was found in the diagnosis of SH.

In overt hypercortisolism, MSC has such a great sensitivity and specificity (greater than 90–95%) that Findling and coauthors proposed its use for screening patients with CS (27).

Conversely, the main result of this study is that late-night salivary cortisol can only suggest the presence of SH and it cannot replace the other conventional endocrine tests such as 24-h UFC, 1 mg overnight DST, and ACTH levels. On the other hand, normal MSC levels cannot exclude hypercortisolism.

It is known that diagnosis of SH is often difficult, due to several factors: absence of specific clinical manifestations lack of a gold standard endocrine test or combination of tests, and controversial diagnostic criteria. Indeed, in order to ascertain this subtle cortisol excess, a series of endocrine tests that are commonly used to diagnose overt hypercortisolism is required (3). Moreover, patients with AI present a fluctuation in cortisol secretion, which makes diagnosis even more difficult (28, 29). This explains why in clinical practice a patient may be diagnosed as not affected by SH at first visit, while they may be classified as SH + in subsequent follow-up or vice versa.

This study suffers from some limitations. First, the absence of a clear biochemical definition of SH may have influenced our results. Secondly, another possible limit of this study is that salivary cortisol was evaluated in only one specimen. The fluctuation in cortisol secretion may explain the limited utility of this approach in the diagnosis of SH. Indeed, in a study of Yaneva et al. (21), five outpatients with proved or suspected overt hypercortisolism, SH, and pituitary masses were evaluated with repeated MSC determinations over several weeks. The only patient with SH provided 16 samples on 16 consecutive days: the mean MSC levels were under the upper cut-off chosen to discriminate overt hypercortisolism and controls, but the 45.8% of the values were above this cut-off (21). Thus, it is possible to hypothesize that in our study a unique salivary cortisol determination could not have been sufficient to disclose a condition of subtle cortisol excess. Moreover, it is not possible to exclude that subsequent determinations of MSC during the follow-up of patients with AI could give more information about the possible presence of an autonomous cortisol secretion.

Although patients with SH do not show the classical manifestations of overt cortisol excess, it is thought that their chronic subtle glucocorticoid excess exposure can lead to detrimental effects such as osteoporosis (11–14), diabetes mellitus, hypertension, dyslipidemia, and insulin resistance (9, 15, 30). In the present study, we did not find differences in the prevalence of these complications between patients with and without SH. In our opinion, the sample we studied may have not been enough large to evidence these differences.

The delayed diagnosis of SH could impact negatively on morbidity and the identification of patients with SH may suggest in some of them the need of adrenalectomy, which has been shown to ameliorate cardiovascular risk profile of these patients (30, 31).

The necessity of using different endocrine tests due to the lack of one gold standard screening test for SH is expensive, time-consuming, and cumbersome. Thus, the identification of a screening method is required. 

### Table 3: Specificity and sensitivity of late-night salivary cortisol levels for the diagnosis of subclinical hypercortisolism.

<table>
<thead>
<tr>
<th>Patients with SH (n=22)</th>
<th>Patients without SH (n=81)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of patients</strong></td>
<td><strong>Number of patients</strong></td>
</tr>
<tr>
<td><strong>MSC &gt; 5.1 nmol/l (%)</strong></td>
<td><strong>MSC &lt; 5.1 nmol/l (%)</strong></td>
</tr>
<tr>
<td>5 (22.7)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 (12.3)</td>
</tr>
<tr>
<td>17 (77.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71 (87.7)</td>
</tr>
</tbody>
</table>

MSC, salivary cortisol at 2300 h. 5.1 nmol/l corresponds to 95th percentile value of our reference population. Subclinical hypercortisolism (SH) was diagnosed if two of the following criteria were present: cortisol after 1 mg overnight dexamethasone suppression test > 83 nmol/l, 24-hour urinary-free cortisol greater than 193 nmol/24 h, and morning ACTH levels < 2.2 pmol/l.

<sup>a</sup>Sensitivity.
<sup>b</sup>Specificity.
this regard, MSC has a poor sensitivity, which does not consent its use as a screening test. However, considering that salivary cortisol is an easy-to-perform and time-saving method, further studies are needed to test the usefulness of repeating its measurement in consecutive days in order to detect the possible cortisol fluctuations that are often present in SH.

In conclusion, in our patients with AI, MSC was found to have a sensitivity of 22.7% and a specificity of 87.7% in the diagnosis of SH. Studies evaluating patients with SH over time with repeated late-night salivary cortisol determinations could clarify the possible role of MSC in the diagnosis of SH.

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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