The role of salivary cortisol measured by liquid chromatography–tandem mass spectrometry in the diagnosis of subclinical hypercortisolism

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Abstract

Objective: The use of late-night salivary cortisol (LNSalC) for diagnosing subclinical hypercortisolism (SH) is debated. No data are available regarding the role of LNSalC as measured by liquid chromatography–tandem mass spectrometry (LC–MS/MS) in SH diagnosis. The aim of this study was to evaluate the diagnostic accuracy of LNSalC measured by LC–MS/MS in SH.

Design: Cross-sectional prospective study of outpatients.

Methods: In 70 consecutive patients with adrenal incidentalomas (AI), without signs and symptoms of hypercortisolism, we diagnosed SH in the presence of at least two of the following: cortisol after 1 mg overnight dexamethasone suppression test (1 mg DST) > 83 nmol/l, 24-h urinary free cortisol (UFC) > 193 nmol/24 h, and morning ACTH < 2.2 pmol/l. The LNSalC levels by LC–MS/MS at 2300 h (normal values < 2.8 nmol/l) and the presence of hypertension, type 2 diabetes mellitus (T2DM), and osteoporosis (OP) were assessed.

Results: The increased LNSalC levels (> 2.8 nmol/l) had an 83.3% specificity (SP) and a 31.3% sensitivity (SN) for predicting the biochemical diagnosis of SH. The increased LNSalC had an 85.2% SP and a 55.6% SN for predicting the presence of hypertension, type 2 diabetes mellitus (T2DM), and osteoporosis (OP) were assessed. The combination of LNSalC > 1.4 nmol/l (cutoff with 100% SN) plus 1 mg DST > 50 nmol/l had an 88.9% SN and an 85.2% SP (similar to SH criterion at enrollment).

Conclusions: In AI patients, LNSalC measured by LC–MS/MS appears to be useful in combination with 1 mg DST for diagnosing SH, while it is not useful as a single criterion.

Introduction

Subclinical hypercortisolism (SH) is defined as a status of altered hypothalamic–pituitary–adrenal (HPA) axis secretion in the absence of the classical signs or symptoms of overt cortisol excess (1, 2). In recent years, SH has become a topic of growing interest, first because of its estimated high prevalence, which is thought to be between 0.2 and 2.0% (3). Indeed, SH is found in 5–30% of patients with incidentally discovered adrenal masses (adrenal incidentalomas (AI)) (3, 4), which, in turn, are thought to be present in up to 4–7% of adults (3). The second reason for the growing interest in SH is that several evidences suggest that this condition may lead to long-term consequences of cortisol excess (i.e. diabetes, hypertension, and osteoporosis (OP)) (5, 6, 7, 8, 9), which may improve after the recovery (3, 10, 11).

However, the correct diagnosis of SH is still a challenge for clinicians (3, 12, 13). The difficulties are related to the lack of specific signs and symptoms of glucocorticoid excess in these patients and, consequently, of a clinical ‘gold standard’. Moreover, the reliability of the commonly used parameters of cortisol secretion in the condition of a subtle glucocorticoid excess is lower than in the overt form of endogenous hypercortisolism (Cushing’s syndrome) (2, 3, 14).

In this light, the determination of late-night serum cortisol levels has been suggested to be useful. However, as its use is limited by the need of hospital admission (15, 16), the usefulness of the more feasible late-night salivary cortisol (LNSalC) has been investigated. In SH, the LNSalC seems to be of limited utility (17, 18, 19, 20, 21, 22) at variance with Cushing’s syndrome (2, 3, 19). These unsatisfactory results may be not only due to the mild degree of cortisol secretion in SH but also due to the diagnostic performance of the laboratory methods (23). The use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) for measuring LNSalC may represent a possible improvement in the SH diagnosis (24, 25, 26, 27). To date, no data are available...
regarding the possible usefulness of LNSaLC measured by LC–MS/MS in SH. Therefore, the aim of this study was to test the diagnostic accuracy of LNSaLC in diagnosing SH.

Materials and methods

Subjects

Between January 2010 and December 2011, 70 (41 females and 29 males) consecutive AI patients referred to our center were included in the study. The inclusion criteria were the following: absence of depression and alcoholism, no diseases and administration of drugs influencing cortisol and dexamethasone metabolism or cortisol secretion, no signs or symptoms of cortisol excess (i.e. moon facies, striae rubrae, skin atrophy, or buffalo hump), no evidence of metastatic disease, and good glycometabolic control (i.e. HbAlc ≤ 7.0%).

All AI were discovered by abdominal ultrasound or computed tomography (CT) scan, performed for the evaluation of unrelated diseases. The finding of AI by ultrasound was confirmed with CT scan. At CT, all adrenal masses were unilateral, homogeneous, and hypodense and with well-shaped features, consistent with the diagnosis of an adrenocortical adenoma. In all patients, the diagnosis of pheochromocytoma and aldosteronoma was excluded by appropriate hormonal determinations (24-h urinary metanephrines and upright plasma renin activity and aldosterone). Written informed consent was obtained from all subjects and the study was approved by our ethics committee. In all patients, the presence of the possible consequences of cortisol excess such as arterial hypertension (AH), type 2 diabetes mellitus (T2DM), and OP was assessed. Subjects with systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg and/or on antihypertensive treatment were defined as affected with AH (28).

Diabetes mellitus was diagnosed using WHO criteria (29). We defined patients as affected with impaired glucose metabolism (IGM) in the presence of T2DM or impaired fasting glucose and/or impaired glucose tolerance.

OP was diagnosed by measuring bone mineral density (BMD) at spine and femur and assessing the presence of vertebral deformities by performing conventional spinal radiographs. The patients were diagnosed as affected with OP in the presence of a BMD T-score (number of S.D. above or below the mean for healthy sex- and ethnicity-matched young adults) at any site lower than −2.5 and/or of vertebral deformities (30).

In all patients, we measured 24-h urinary free cortisol (UFC, normal values: 27.6–193 nmol/24 h), plasma ACTH at 0800 h (normal values: 2.2–4.4 pmol/l), serum cortisol levels at 0800 h after 1 mg overnight dexamethasone suppression test (1 mg DST), and LNSaLC at 2300 h (normal values: < 2.8 nmol/l). The upper limit of LNSaLC corresponds to the 95th percentile value of our reference population of normal subjects. This population is formed by 60 healthy subjects (28 males, 32 females, age 40.3 ± 10.8, range 25–69; BMI 24.4 ± 2.8, range 21–31) recruited from the staff of our center.

We classified patients as affected with SH in the presence of at least two of the following three criteria: 1 mg DST > 83 nmol/l, UFC level > 193 nmol/24 h, and morning ACTH levels < 2.2 pmol/l. In SH patients without clearly suppressed ACTH levels (i.e. > 1.1 pmol/l), a CRH stimulation test was performed. The use of a 1 mg DST cutoff of > 83 nmol/l rather than 138 nmol/l as recommended by the National Institutes of Health (31) was preferred to increase the test sensitivity (SN). We use these criteria because they have been previously substantiated on a clinical basis (3). According to the presence of SH, the patients were subdivided in two groups: 16 subjects with SH (Group SH+) and 54 without SH (Group SH−).

Methods

Salivary samples were collected by chewing a cylindrical cotton swab (Salivette, Sarstedt, Nümbrecht, Germany) for about 2 min at 2300 h at home. At least 3 h before the collection, the subjects were told not to eat and brush their teeth. The specimens, collected at home over two consecutive days, were refrigerated at a temperature of 2–8 °C and were brought to the laboratory within 1 day. The received samples were centrifuged at 1207 g for 3 min, the cotton swab was removed, and the collected saliva samples were frozen at −20 °C until assayed. The determination of cortisol by LC–MS/MS was carried out in the presence of cortisol-d4 as deuterated internal standard, after submitting the saliva sample to a purification by an online TurboFlow system (Thermo Scientific, Rodano, Italy), using a Cyclone column Cyclone column (50 mm length, 0.5 mm internal diameter, Thermo Scientific). Cortisol was separated in liquid chromatography using a C18 reversed-phase column (Hypersil Gold, 50 mm length, 2.1 mm internal diameter, and 3 μm particle size, Thermo Scientific), and a gradient of aqueous ammonium formate (5 mM) in 0.1% formic acid and methanol as eluent, flowing at 0.7 ml/min. Detection and quantification were performed by a triple quadrupole mass spectrometer (TSQ Quantum Access, Thermo Scientific) equipped with a heated electrospray ionization source (H-ESI), operating in the positive ion mode. The ionization source parameters were spray voltage 4000 V, ion transfer tube temperature 220 °C, vaporization temperature 202 °C, nitrogen as sheath gas and auxiliary gas operating at the pressure of 50 and 5 units (arbitrary scale), and tube lens offset 94 V. Collision-induced dissociation was performed using
Ar, as the collision gas, at a pressure of 1.5 m Torr. The quantification was based on selective reaction monitoring following the transitions m/z 363→121 (collision energy 24 eV) and m/z 367→121 (collision energy 25 eV) for cortisol and cortisol-d4 respectively. The method had a precision, assessed as percent coefficient of variation, of <5%, an accuracy between 99 and 102%, and a limit of quantification of 0.27 nmol/l. The throughput was about 50 samples/day. The mean value of two different determinations was calculated.

Serum, plasma, and urinary samples were stored at −20 °C until assayed. In all patients, plasma morning ACTH levels (mean of three determinations at 20-min intervals) were measured by IRMA (BRAHMS Diagnostica GmbH, Berlin, Germany), and serum cortisol and UFC levels (after dichloromethane extraction) were determined immunofluorometrically by TDX-FLX Abbott, GmbH, Diagnostika kits, at the study entry. The intra- and interassay coefficients of variation were <15% for ACTH and <10% for serum cortisol and UFC.

In all patients, BMD was measured by dual-energy X-ray absorptiometry (Hologic Discovery. Software version 13.3:3, Watham, MA, USA) at lumbar spine (LS, precision 1.0%) and femoral neck (FN, precision 1.8%). Individual BMD values were expressed as SD units (Z-values) in relation to age-matched reference population (32). Fractured vertebrae were excluded from BMD measurement.

Conventional spinal radiographs (T4-L4) in lateral and anteroposterior projection were obtained with standardized technique in all subjects. Two trained radiologists, who were blinded to BMD and hormonal data, independently reviewed the radiographs. Vertebral fractures were diagnosed on visual inspection using the semiquantitative visual assessment, previously described by Genant et al. (33). According to this technique, fractures assessed on lateral thoracolumbar spine radiographs were defined as reductions of more than 20% in anterior, middle, or posterior vertebral height.

Statistical analysis and design of the study
Statistical analysis was performed by SPSS version 18.0 statistical package (SPSS, Inc.). The results are expressed as mean ± s.d.

The normality of distribution was tested by Kolmogorov–Smirnov test. The comparison of continuous variables between Group SH+ and Group SH− was performed using Student’s t-test or Mann–Whitney U test as appropriate. Categorical variables were compared by χ² test. The associations among the different indexes of cortisol secretion were tested by either Pearson Product Moment correlation or Spearman correlation as appropriate. The SN and specificity (SP) of LNSalC for predicting the biochemical diagnosis of SH and the alteration of the other indices of cortisol secretion were calculated.

As SH is clinically silent, a clinical ‘gold standard’ for diagnosing SH is lacking. However, SH patients often show the concomitant presence of IGM, AH, and OP, which are typical, although not specific, consequences of cortisol excess. Therefore, in order to compare the diagnostic accuracy of LNSalC with the other routinely used parameters of cortisol secretion, we tested the SN, SP, and overall accuracy of LNSalC, 1 mg DST, UFC, and ACTH (individually taken or in combination) in predicting the concomitant presence of IGM, AH, and OP. Moreover, the receiver operator characteristics (ROC) analysis was performed to assess the threshold for LNSalC with the best compromise between SN and SP for predicting the concomitant presence of the three chronic comorbidities. The parameter or combination of parameters with an SN and SP of at least 50% and an overall accuracy of at least 60% were reported. Finally, a chronic comorbidity score was calculated for each patient by summing the number of the three chronic comorbidities of SH among IGM, AH, and OP (range 0–3). General linear modeling was used for comparing the different indexes of cortisol secretion and the LS and FN BMD in SH+ group and SH− group after adjusting for gender. P values of <0.05 were considered significant.

Results
The clinical characteristics of Group SH+ and Group SH− are reported in Table 1. Age, gender, and BMI were comparable between the two groups. The diameter of adenoma was higher in SH+ than in SH− group. LNSalC tended to be higher in SH+ than in SH − group, even if the statistical significance was not reached. Group SH+ had lower LS and FN BMD and higher chronic comorbidity scores and prevalence of vertebral fractures, OP and IGM than Group SH−. In the SH+ group, the number of patients without comorbidities and with three comorbidities was significantly lower and higher respectively than in Group SH−. As the number of female patients tended to be higher in SH− than in SH+ group, even without reaching the statistical significance, we compared 1 mg DST, UFC, ACTH, LNSalC, and Z-score values after adjusting for gender by general linear modeling and found substantially the same results (data not shown).

The patient in the SH+ group with UFC 827.7 nmol/24 h had all three possible complications of SH (AH, OP, and IGM) and LNSalC was 4.3 nmol/l, but no feature of clinical cortisol excess (i.e. purple striae, easy bruising, proximal muscle weakness, and plethora).

As shown in Table 2, the increased LNSalC levels (>2.8 nmol/l, the upper limit of the normal range) showed good SP but low SN for predicting the presence of the biochemical diagnosis of SH as defined at the enrollment and for individuating patients with 1 mg DST levels above 83 nmol/l. The cutoff of LNSalC with...
the 100% SN for predicting the absence of the biochemical diagnosis of SH was ≤1.4 nmol/l.

Bivariate analyses showed that 1 mg DST was inversely correlated with ACTH levels (r = −0.32, P = 0.007) and directly with UFC (r = 0.36, P = 0.002). The LNSalC levels were directly associated with 1 mg DST (r = 0.45, P < 0.0001), while no correlations were found between LNSalC levels and the other parameters of cortisol secretion and adenoma size. The chronic complication score was associated with 1 mg DST and LNSalC levels (r = 0.44, P < 0.0001 and r = 0.37, P = 0.002 respectively).

We used the concomitant presence of the three more frequent comorbidities of SH, as clinical parameter, in order to compare the diagnostic accuracy of 1 mg DST, UFC, ACTH, and LNSalC (individually taken or in combination) for diagnosing SH. The ROC analysis showed that the cutoff level of LNSalC with the best compromise between SN and SP for predicting the concomitant presence of the three comorbidities was 1.93 nmol/l (AUC = 0.814; P = 0.002).

The criteria for predicting the concomitant presence of the three chronic comorbidities with an SN and SP of at least 50% and an overall accuracy of at least 60% are reported in Table 3. The combination of the parameters of cortisol secretion we used at the study entry as the biochemical criterion to diagnose SH (Criterion I) showed a good compromise between SN and SP (88.9 and 86.9% respectively) with good overall accuracy, for predicting the cluster of comorbidities.

In comparison with Criterion I, the LNSalC, with the cutoff corresponding to the upper limit of the normal range (Criterion II), showed a similar SP but a lower SN.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group SH+ (n=16)</th>
<th>Group SH− (n=54)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.5 ± 10.6</td>
<td>61.5 ± 10.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>7/9</td>
<td>34/20</td>
<td>0.17</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.7 ± 4.6</td>
<td>27.1 ± 4.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Diameter of adenoma (cm)</td>
<td>3.2 ± 1.5</td>
<td>2.1 ± 0.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1 mg DST (nmol/l)</td>
<td>90.8 ± 40.8</td>
<td>35.3 ± 16.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ACTH (pmol/l)</td>
<td>1.6 ± 1.0</td>
<td>3.6 ± 2.1</td>
<td>0.001</td>
</tr>
<tr>
<td>UFC (nmol/24 h)</td>
<td>301.3 ± 162.4</td>
<td>149.2 ± 85.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LNSalC (nmol/l)</td>
<td>2.29 ± 1.65</td>
<td>1.69 ± 1.23</td>
<td>0.107</td>
</tr>
<tr>
<td>BMD Z-score (0–3)</td>
<td>−0.66 ± 1.4</td>
<td>−0.81 ± 1.5</td>
<td>0.001</td>
</tr>
<tr>
<td>FN BMD Z-score (0–3)</td>
<td>−1.03 ± 1.1</td>
<td>0.6 ± 0.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Patients with vertebral fractures</td>
<td>11 (68.8%)</td>
<td>10 (18.5%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Patients with OP</td>
<td>14 (87.5)</td>
<td>15 (27.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Patients with hypertension</td>
<td>11 (68.8)</td>
<td>32 (59.3)</td>
<td>0.493</td>
</tr>
<tr>
<td>Patients with impaired glucose metabolism</td>
<td>11 (68.8)</td>
<td>17 (31.5)</td>
<td>0.01</td>
</tr>
<tr>
<td>0 complications</td>
<td>0 (0)</td>
<td>12 (22.2)</td>
<td>0.05</td>
</tr>
<tr>
<td>1 complication</td>
<td>4 (25)</td>
<td>21 (38.9)</td>
<td>0.38</td>
</tr>
<tr>
<td>2 complications</td>
<td>4 (25)</td>
<td>20 (37)</td>
<td>0.55</td>
</tr>
<tr>
<td>3 complications</td>
<td>8 (50)</td>
<td>1 (1.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Score of chronic complications</td>
<td>2.25 ± 0.9</td>
<td>1.19 ± 0.8</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

1 mg DST, serum cortisol after 1 mg overnight dexamethasone suppression test (1 mg DST); UFC, 24-h urinary free cortisol; LNSalC, salivary cortisol at 2300 h (normal values <2.8 nmol/l); BMD, bone mineral density; LS, lumbar spine; FN, femoral neck; IGM, impaired glucose metabolism; AH, arterial hypertension; OP, osteoporosis.

The use of the cutoff obtained by the ROC analysis (Criterion III) increased the SN (77.8%) but lowered the SP. The cutoff for LNSalC with a 100% SN for predicting the absence of the comorbidities was ≤1.4 nmol/l (Criterion IV).

The addition of LNSalC to the parameters included in the Criterion I (1 mg DST, ACTH, and UFC, at least two altered parameters out of four, Criterions V, VI, and VII) did not give greater advantage than Criterion I itself. Similarly, in the presence of both LNSalC levels >1.93 nmol/l and Criterion I (Criterion VIII), the accuracy and SP increased, but SN decreased compared with Criterion I alone. The Criterion X characterized by the absence of ACTH, which is replaced by LNSalC.

<table>
<thead>
<tr>
<th>Parameters of HPA axis activity</th>
<th>SN</th>
<th>SP</th>
<th>Accuracy</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>At least two</td>
<td>31.3</td>
<td>83.3</td>
<td>71.4</td>
<td>0.02</td>
</tr>
<tr>
<td>out of 1 mg DST</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 83 nmol/l, increased</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UFC, low ACTH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg DST &gt; 50 nmol/l</td>
<td>57.1</td>
<td>69.6</td>
<td>67.1</td>
<td>0.06</td>
</tr>
<tr>
<td>1 mg DST &gt; 83 nmol/l</td>
<td>35.7</td>
<td>89.3</td>
<td>78.5</td>
<td>0.036</td>
</tr>
<tr>
<td>1 mg DST &gt; 138 nmol/l</td>
<td>14.3</td>
<td>100</td>
<td>82.8</td>
<td>0.038</td>
</tr>
<tr>
<td>Increased UFC</td>
<td>28.6</td>
<td>66.1</td>
<td>58.6</td>
<td>0.484</td>
</tr>
<tr>
<td>Low ACTH</td>
<td>50</td>
<td>60.7</td>
<td>58.5</td>
<td>0.467</td>
</tr>
</tbody>
</table>

1 mg DST, serum cortisol after 1 mg overnight dexamethasone suppression test (1 mg DST); UFC, 24-h urinary free cortisol; Increased UFC, >193 nmol/24 h; Low ACTH, <2.2 pmol/l; Increased LNSalC, salivary cortisol at 2300 h >2.8 nmol/l.
and by the presence of also increased UFC and 1 mg DST > 50 nmol/l had a similar diagnostic accuracy to Criterion I.

As expected, the use of 1 mg DST alone with the cutoff used for the screening of overt hypercortisolism (50 nmol/l, Criterion XII) showed good SN (88.9%) but an unsatisfactory SP (72.1%). At variance, the combination of LNSalC > 1.4 nmol/l and 1 mg DST > 50 nmol/l (Criterion XIII) predicted the presence of the comorbidities with a similar diagnostic accuracy to Criterion I (Fig. 1).

Finally, we tested the diagnostic accuracy of the Criterion I in the subgroup of patients \( n = 36 \) with LNSalC levels 1.4 nmol/l (the cutoff with 100% SN) and found that in this subgroup the Criterion I had a 88.9% SN, SP, and accuracy \( P < 0.0001 \). The use of this protocol (Criterion I only in patients with LNSalC > 1.4 nmol/l) correctly predicted the absence or presence of the three comorbidities in 66 out of the 70 patients (accuracy 94.3%).

### Discussion

To our knowledge, this is the first study evaluating the usefulness of LNSalC assessed by LC–MS/MS in the diagnosis of SH, in comparison with the other routinely used parameters of cortisol secretion. We tested the diagnostic accuracy of LNSalC in predicting the biochemical diagnosis of SH, as established by a commonly used criterion. Then, in order to compare the diagnostic accuracy of LNSalC with the other biochemical parameters of cortisol secretion (1 mg DST, ACTH, and UFC, individually taken or in combination), we tested their usefulness in predicting the concomitant presence of the three more common comorbidities of SH.

We found that the increased LNSalC as single parameter had a good SP but low SN for predicting the presence of SH, as established by the commonly used biochemical parameters (Table 2). Similarly, LNSalC had a good SP but low SN in predicting the concomitant

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**Table 3** Diagnostic accuracy of late-night salivary cortisol measured by tandem mass spectrometry (LNSalC) and of other parameters and combinations of parameters of pituitary–adrenal axis activity in predicting the concomitant presence of IGM, AH, and OP. Data are expressed as percentages.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Parameters</th>
<th>SN</th>
<th>SP</th>
<th>Accuracy</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2 out of: 1 mg DST &gt; 83 nmol/l, increased UFC, low ACTH</td>
<td>88.9</td>
<td>86.9</td>
<td>81.4</td>
<td>0.008</td>
</tr>
<tr>
<td>II</td>
<td>Increased LNSalC</td>
<td>55.6</td>
<td>85.2</td>
<td>81.4</td>
<td>0.013</td>
</tr>
<tr>
<td>III</td>
<td>LNSalC &gt; 1.93 nmol/l ( \mu g/dl ) (^a)</td>
<td>77.8</td>
<td>68.9</td>
<td>70</td>
<td>0.011</td>
</tr>
<tr>
<td>IV</td>
<td>LNSalC ≤ 1.4 nmol/l ( \mu g/dl ) (^a)</td>
<td>100</td>
<td>55.7</td>
<td>62</td>
<td>0.002</td>
</tr>
<tr>
<td>V</td>
<td>2 out of SeDST &gt; 50 nmol/l, increased UFC, low ACTH, increased LNSalC</td>
<td>88.9</td>
<td>68.9</td>
<td>71.4</td>
<td>0.002</td>
</tr>
<tr>
<td>VI</td>
<td>2 out of SeDST &gt; 83 nmol/l, increased UFC, increased LNSalC</td>
<td>88.9</td>
<td>82</td>
<td>82.9</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>VII</td>
<td>2 out of SeDST &gt; 83 nmol/l, low ACTH, LNSalC &gt; 1.93 nmol/l (^a)</td>
<td>88.9</td>
<td>75.4</td>
<td>77</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>VIII</td>
<td>Criterion I plus LNSalC &gt; 1.93 nmol/l (^a)</td>
<td>66.7</td>
<td>96.7</td>
<td>92.8</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>IX</td>
<td>2 out of SeDST &gt; 50 nmol/l, increased UFC, increased LNSalC</td>
<td>77.8</td>
<td>86.9</td>
<td>85.7</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>X</td>
<td>2 out of SeDST &gt; 50 nmol/l, increased UFC, LNSalC &gt; 1.93 nmol/l (^a)</td>
<td>88.9</td>
<td>83.6</td>
<td>84.2</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>XI</td>
<td>2 out of SeDST &gt; 83 nmol/l, increased UFC, LNSalC &gt; 1.93 nmol/l (^a)</td>
<td>66.7</td>
<td>86.9</td>
<td>84.2</td>
<td>0.001</td>
</tr>
<tr>
<td>XII</td>
<td>1 mg DST &gt; 50 nmol/l</td>
<td>88.9</td>
<td>72.1</td>
<td>68.5</td>
<td>0.001</td>
</tr>
<tr>
<td>XIII</td>
<td>1 mg DST &gt; 50 nmol/l plus LNSalC &gt; 1.4 nmol/l (^b)</td>
<td>88.9</td>
<td>85.2</td>
<td>85.7</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

IGM, impaired glucose metabolism; AH, arterial hypertension; OP, osteoporosis; 1 mg DST, serum cortisol after 1 mg overnight dexamethasone suppression test (1 mg DST); UFC, 24-h urinary free cortisol; Increased UFC, > 193 nmol/24 h; Low ACTH, < 2.2 pmol/l; Increased LNSalC, salivary cortisol at 2300 h > 2.8 nmol/l.

\(^a\)Cutoff obtained by ROC analysis.

\(^b\)Cutoff with a 100% SN.

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**Figure 1** Correlation between late-night salivary cortisol (LNSalC) and cortisol after 1 mg overnight dexamethasone suppression test (1 mg DST) levels. The closed diamond symbol indicates patients with the concomitant presence of impaired fasting glucose, arterial hypertension (AH), and osteoporosis (OP). The open diamond symbol indicates patients without the concomitant presence of impaired fasting glucose, AH, and OP. The vertical line represents the cutoff of salivary cortisol at 2300 h (LNSalC) with 100% sensitivity for predicting the concomitant presence of AH, impaired glucose metabolism, and OP. The horizontal line corresponds to the commonly used cutoff of cortisol after 1 mg overnight dexamethasone suppression test (1 mg DST) for the screening of overt hypercortisolism. The LNSalC levels were directly associated with the presence of also increased UFC and 1 mg DST > 50 nmol/l predicted the concomitant presence of impaired fasting glucose, AH, and OP with a 85.2% SP, 88.9% SN, and 85.7% accuracy \( P < 0.0001 \).
presence of AH, IGM, and OP (Table 3). However, the use of LNSalC in combination with 1 mg DST (with the lowest cutoffs) showed a diagnostic accuracy similar to that of the routinely used combination of parameters for diagnosing SH (Fig. 1). Finally, the use of LNSalC as screening test, followed by Criterion I only in patients who tested positive to LNSalC, showed an SN, SP, and accuracy of almost 90%.

Several studies showed that, at variance with Cushing’s syndrome (2, 3, 19, 34), in SH, the LNSalC measured by immunoassay has been reported to be of limited utility (19, 20, 21, 22, 23), with the exception of the study of Tateishi et al. (35) who found a 94–100% SN. These discordances may be due to the different criteria used for diagnosing SH and to the different immunoassay methods used in the various studies. Overall, these generally unsatisfactory findings may be explained by the fact that in SH patients, the degree of cortisol secretion is lower than in CS ones and may be fluctuant (1, 3). Therefore, the lower pretest probability of the disease in patients with SH (due to the absence of clinical signs and symptoms) leads to a reduction of the diagnostic accuracy of all parameters of cortisol secretion (14). However, the unsatisfactory results of employing LNSalC for diagnosing SH may also be due to the diagnostic performance of the various laboratory methods (23). In the last years, the LNSalC levels measured by LC–MS/MS have been found to have a 83–100% SN for identifying patients with CS (24, 25, 26, 27, 36). No study has evaluated the possible role of LNSalC measured by LC–MS/MS in the diagnosis of SH.

From the present data, in patients with AI, LNSalC measured by LC–MS/MS cannot be used as a single parameter for screening for SH due to the low SN. This is in keeping with previous data showing that LNSalC determination alone is not a reliable tool for the screening of SH (19, 20, 21). However, the novel finding is that the combination of LNSalC measured by LC–MS/MS and 1 mg DST might be reliably used for diagnosing SH (Fig. 1), without the need of the measurement of UFC and ACTH. This is of importance as the determination of UFC is cumbersome and the ACTH measurement is often not fully reliable. Therefore, the possible use of the simple determination of LNSalC and 1 mg DST may consent to more easily investigate the possible presence of SH in AI patients.

The fact that the determination of ACTH is not necessary in the presence of the measurement of LNSalC by LC–MS/MS has another advantage. Indeed, it could be possible to investigate the presence of SH even in patients with a possible ACTH-dependent form (3). In this condition, the use of the ACTH is known to be useless for screening purposes (3). On the contrary, once the diagnosis of SH is established, the determination of the ACTH levels is mandatory in order to exclude an ACTH-dependent form of SH (3). Further studies are needed to investigate the possible role of LNSalC measured by LC–MS/MS in the screening of the ACTH-dependent SH.

The diagnosis of SH remains, to date, a challenge for the physicians, as the need for high SP (to avoid too many false positives) has not consented to have biochemical criteria with enough SN. Indeed, the few available intervention studies suggest that the comorbidities of SH may improve after surgery also in some AI patients without a presurgical biochemical evidence of SH (3, 7, 11). It is likely, therefore, that some patients diagnosed as not affected with SH have, in fact, a subtle degree of hypercortisolism and must be considered false negatives to the biochemical screening. Therefore, to date, we are still looking for biochemical parameters with enough SN and SP for diagnosing SH. For this reason, to further increase the diagnostic accuracy and simplify the biochemical work-up, this study suggests a possible protocol. Indeed, the use of LNSalC as a screening test and subsequently of Criterion I only in patients with LNSalC > 1.4 nmol/l correctly predicts the absence or presence of the three comorbidities in 94.3% of patients. This approach may consent to reserve the more cumbersome tests to only 50% of AI patients.

The limits of this study are related, first, to its cross-sectional design that consent to investigate associations but not causality. Indeed, the lack of a prospective intervention arm of the study precludes the possibility to validate the diagnosis of SH made by LNSalC measured by LC–MS/MS with clinical endpoints (i.e. improving or worsening of the chronic complications of SH). Moreover, in this group of patients, we did not evaluate the salivary cortisol by immunoassay to compare the diagnostic accuracy of the two methods in the same patients. This comparison might have been useful as some studies on overt CS reported a similar performance of LNSalC measured by LC–MS/MS than by immunoassay, which, however, has the advantage of simplicity (37). In addition, in our methods, a cotton swab was used. This could be an important issue because a significantly lower recovery of salivary steroids using a cotton swab compared with polyester swab has been reported. In particular, the salivary sample collected by a cotton swab has been shown to be not fully reliable over physiological concentrations (38). A possible methodological limitation of the study is that the number of subjects that make up the control group (60 healthy subjects) could be too small to accurately evaluate the diagnostic accuracy and performance of a complex procedure like LC–MS/MS. Another limit is related to the disease per se. Indeed, in AI patients, cortisol secretion has a highly variable pattern and, therefore, diagnosing SH by establishing arbitrary cutoffs of indexes of cortisol secretion leads to unavoidable mistakes in classifying some patients (3). In addition, the lack of a clinical picture specific for SH leads to a reduction of pretest probability of the disease and, therefore, of the diagnostic accuracy of all...
parameters of cortisol secretion (14). However, our approach that considered the concomitant presence of AH, IGM, and OP as suggestive for SH may have led to increase the pretest probability of the disease. Validating the criteria for diagnosing ‘SH’ on clinical grounds might be considered paradoxical. Nowadays, however, ‘SH’ is considered probably not ‘subclinical’, as the recovery from this condition seems to improve the chronic comorbidities (3, 7, 10, 11). Therefore, with the lack of other possible ‘gold standard’ parameters, investigating the accuracy of the different SH criteria in predicting the combination of the possible chronic comorbidities of SH seems a reasonable approach.

In conclusion, notwithstanding these limits, this study suggests that in AI patients, LNSalC measured by LC–MS/MS: i) cannot be used as a single parameter for the screening of SH; ii) can be used together with 1 mg DST for diagnosing SH with an accuracy comparable to that of the routinely used biochemical parameters; and iii) may consent to select the subjects, in whom the determination of other parameters of cortisol secretion is mandatory.

**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.

**Funding**

This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

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Received 11 September 2012
Revised version received 26 November 2012
Accepted 3 December 2012