Clinical utility of late-night and post-overnight dexamethasone suppression salivary cortisone for the investigation of Cushing’s syndrome

CM Ng *, TK Lam, YC Au Yeung, CH Choi, YP Iu, CC Shek, SC Tiu

ABSTRACT

Introduction: There is a pressing need to identify diagnostic testing for Cushing’s syndrome that can be achieved with ease and at low cost. This study aimed to explore the usefulness of late-night and post-overnight 1-mg dexamethasone suppression salivary cortisone, as measured by liquid chromatography–tandem mass spectrometry, for investigation of hypercortisolism.

Methods: Salivary cortisone data of subjects were investigated according to a pre-specified protocol. Subjects were classified as having ‘hypercortisolism’ or ‘eucortisolism’ on the basis of histological or biochemical criteria. Receiver operating characteristic curves were drawn to identify the cut-off values and study their performance characteristics. We measured 24-hour urinary free cortisol; late-night salivary cortisol and cortisone; and post-overnight 1-mg dexamethasone suppression serum cortisol, and salivary cortisol and cortisone. Saliva and urine samples were assayed by liquid chromatography–tandem mass spectrometry.

Results: In this study, 21 subjects were classified as having hypercortisolism and 78 as having eucortisolism. A late-night salivary cortisone cut-off of 13.50 nmol/L had a sensitivity of 94.7% and a specificity of 87.2%. After taking 1-mg dexamethasone the night before, a salivary cortisol cut-off of 0.85 nmol/L had a sensitivity of 76.2% and a specificity of 96.2%; a salivary cortisone cut-off of 7.45 nmol/L had a sensitivity of 85.7% and a specificity of 94.9%, while a salivary cortisone cut-off of 3.25 nmol/L had a sensitivity of 95.2% and a specificity of 79.5%. Many salivary cortisol samples were below the detection limit of liquid chromatography–tandem mass spectrometry. In comparison with salivary cortisol, salivary cortisone had a better correlation with total serum cortisol and better diagnostic performance following dexamethasone suppression.

Conclusions: Both late-night and post-overnight dexamethasone suppression salivary cortisone levels are of diagnostic value in the investigation of hypercortisolism.

New knowledge added by this study
- Compared with salivary cortisol, salivary cortisone has better diagnostic performance after dexamethasone suppression.
- Both late-night and post-overnight dexamethasone suppression salivary cortisone levels are of diagnostic value in the investigation of hypercortisolism.

Implications for clinical practice or policy
- Using the cut-off value generated from this study, late-night and post-overnight dexamethasone suppression salivary cortisone, measured by liquid chromatography–tandem mass spectrometry, can be added to the panel of diagnostic tests for hypercortisolism.
Methods

Subjects

All subjects referred to the Endocrine Unit of Queen Elizabeth Hospital in Hong Kong for suspected endogenous hypercortisolism were evaluated according to a pre-specified protocol. Written informed consent was obtained from all subjects. The results of subjects investigated during May 2013 (when salivary measurement by LC-MS/MS became available) to September 2016 were reviewed. This study was approved by the Hospital Ethics Committee. No patient was receiving medical treatment for Cushing’s syndrome at the time of assessment. Subjects who were taking medication (such as rifampicin, phenytoin, phenobarbital, and alcohol) that might interfere with dexamethasone metabolism, or were night or shift workers, were excluded.

Investigations performed

Detailed oral and written instructions were given to all subjects by an endocrine specialist nurse. For the collection of salivary sample, subjects were instructed to refrain from smoking, brushing teeth, and eating or drinking anything but water for at least 30 minutes before collection. Saliva samples were collected using a cotton swab in Salivette tubes (Sarstedt, Nümbrecht, Germany). Salivettes were kept at 4°C in a home refrigerator and sent to the laboratory within 24 hours.

According to the pre-specified protocol, on day 1, a 24-hour urine sample was collected for UFC measurement. On day 2, a 0900h salivary sample was collected at the Endocrine Centre, under nurse supervision. The patient was then instructed to collect a late-night (between 2300h and 2400h) saliva sample that evening, after which he/she was to take dexamethasone 1 mg orally. On day 3, subjects were instructed to refrain from smoking, brushing teeth, and eating or drinking anything but water for at least 30 minutes before collection. Saliva samples were collected using a cotton swab in Salivette tubes (Sarstedt, Nümbrecht, Germany). Salivettes were kept at 4°C in a home refrigerator and sent to the laboratory within 24 hours.

Laboratory assays

Serum cortisol was measured by competitive
chemiluminescent microparticle immunoassay using the Abbott ARCHITECT i2000SR system (Abbott Diagnostics, Illinois, US). The coefficient of variation of the assay for serum cortisol was 4.0%-6.2% at low levels and 3.3%-4.3% at high levels. Salivary cortisol and salivary cortisone were measured by LC-MS/MS using the Waters Xevo TQ MS system (Waters Corporation, Milford [MA], US). Cortisol and cortisone were extracted from saliva using the organic solvent methyl-tert-butyl-ether after addition of a deuterium-labelled internal standard mixture of cortisol-d4 and cortisone-d8 (CDN isotopes). The organic supernatant was dried under nitrogen at a temperature below 45°C and dissolved in the initial mobile phase for LC-MS/MS analysis. The steroid analytes were separated from the matrix background in a reversed-phase chromatography that employed a sub-2 μm analytical column (ACQUITY UPLC HSS T3 Column, 2.1 x 100 mm, 1.8 mm; Waters Corporation, Milford [MA], US) and a 6-minute elution method consisting of a gradient mixture of 0.1% glacial acetic acid, 0.2 mM ammonium acetate in water and methanol. Negative electrospray mode was used for analyte ionisation. The charged acetate adducts were monitored by multiple reaction monitoring mode with two stable mass transitions for cortisol (421>331; 421>297) and cortisone (419>329; 419>301) and one multiple reaction monitoring for each of the corresponding deuterated internal standards (cortisol-d4: 425>335; cortisone-d8: 427>337). Quantitative measurement was derived using the linear least squares regression method with origin excluded and 1/x weighting for better accuracy at a low concentration level. The coefficient of variation of the assay for salivary cortisol and cortisone was 5%-7% and 7%-10%, respectively across the analyte reporting ranges up to 250 nmol/L. The lower limit of detection was 0.5 nmol/L for both salivary cortisol and cortisone. Urinary cortisol was also measured by LC-MS/MS. Adrenocorticotropic hormone (ACTH) was measured by Immulite 2000 XPi (Siemens Healthcare GmbH, Erlangen, Germany) chemiluminescent immunometric assay. The upper reference limit of ACTH was 10.2 pmol/L.

Definition of hypercortisolism

Subjects were classified as having hypercortisolism if either the biochemical or the histological criterion was fulfilled. The biochemical criterion was defined as having an abnormal value in at least two of the following three tests: (1) SerFDex >138 nmol/L, or >50 nmol/L in the context of adrenal incidentaloma15; (2) UFC >157 nmol/day; and (3) SalF LN ≥3 nmol/L. The categorisation of hypercortisolism was made without knowledge (ie blinded) of the three outcome parameters being evaluated for diagnostic accuracy, namely SalE LN, SalF LN, and SalE LN. The reference range for UFC in our laboratory, derived locally from the 2.5th to 97.5th percentile of 112 healthy adults, was 22-157 nmol/day. The reference level for SalF LN in our laboratory, derived from the 97.5th percentile of 61 normal individuals, was <3 nmol/L. The histological criterion was defined as histological proof and postoperative improvement in biochemical and clinical parameters. Subjects not fulfilling either of these criteria were classified as having eucortisolism.

Statistical analyses

For calculation purposes, results below the detection limit of the assay were set to the lowest detection value. Continuous data were expressed as mean ± standard deviation if parametric, and median (range) if non-parametric. Chi squared test was used to detect any difference between categorical data. Unpaired t-test was used to compare continuous variables, and Mann-Whitney test was used for non-parametric data. A P value of <0.05 was considered statistically significant. Correlation between serum and salivary values were performed using Pearson correlation coefficients.

For estimation of the optimal diagnostic cut-off value, receiver operating characteristic (ROC) curves were drawn using data from the subjects classified as hypercortisolism or eucortisolism. The optimal cut-off was chosen where the Youden’s index (sensitivity + specificity -1) was maximal. The test performance characteristics were calculated to assess their utility. The quality of diagnostic tests was expressed as the area under ROC curve (AUC). For sample size requirement estimation, for an estimated AUC of 0.8, a minimum of nine positive cases was required.16 Statistical analysis was performed using the Statistical Package for Social Science (Windows version 20.0; IBM Corp, Armonk [NY], US).

Results

A total of 115 subjects were referred to our Endocrine Clinic for assessment of hypercortisolism during the study period. Of them, 14 subjects with a history of transsphenoidal surgery or adrenalectomy who had been referred for postoperative assessment of endocrine function were excluded. One patient with ongoing investigations and pending re-evaluation and another with end-stage renal failure were also excluded. No patient was taking exogenous steroids. All subjects had normal renal and liver function tests.

A total of 115 sets of biochemical investigations were performed in 99 subjects (40 males, 59 females; mean age, 55.3 ± 14.3 years; range, 19-81 years). The primary indications for testing were adrenal incidentaloma in 52 subjects, suspected secondary hypertension or diabetes mellitus in 25, Cushingoid features in 21, and pituitary mass in one. Eleven subjects had two or more sets of investigations.
performed (two patients had 4 sets, one patient had 3 sets, and eight patients had 2 sets). For these subjects, only the data set with the highest SerFDex was chosen for analysis. In two subjects, the volume of the late-night salivary sample was inadequate for measurement.

In this study, 21 subjects were found to have hypercortisolism according to the above criteria—20 subjects met the biochemical criterion; eight subjects met the histological criterion, including one whose set of tests did not fulfil the biochemical criterion (SerFDex 135 nmol/L; normal UFC and SalFLN, ACTH 1.1 pmol/L) and who underwent surgery because of an adrenal adenoma that enlarged from 1.6 cm to 2.5 cm over 2 years, postoperative spot cortisol was <28 nmol/L and hydrocortisone replacement was required for 6 months before axis recovery. Among the 21 subjects who had hypercortisolism, 7 had adrenal Cushing’s, 4 pituitary Cushing’s, 3 ectopic ACTH syndrome, 1 adrenocortical carcinoma, and 6 subclinical Cushing’s. Of these subjects, 14 (67%) had elevated UFC, 18 (86%) had non-suppressed SerFDex, and 17 (81%) had elevated SalFLN. Among the eight subjects with histological proof (6 adrenalectomies, 1 transsphenoidal surgery, 1 enucleation of pancreatic neuroendocrine tumour), all had clinical and biochemical improvement after operation. Eucortisolism was diagnosed in 78 subjects according to the aforementioned criteria.

The baseline characteristics of the subjects are shown in Table 1. There were no statistically significant differences between the hypercortisolism and the eucortisolism groups with respect to age, gender, and prevalence of obesity, diabetes mellitus, or hypertension. There was a statistically significant higher prevalence of Cushingoid features and of proximal myopathy in the hypercortisolism group.

The biochemical test results are shown in Table 2. The hypercortisolism group had statistically significant higher levels of SerFDex, UFC, SalFLN, SalFDex, and SalFDex. No SalFLN sample in the hypercortisolism group and 17 (21.8%) SalFLN samples in the eucortisolism group had levels below the detection limit of 0.5 nmol/L. No SalELN sample in the hypercortisolism group had levels below the detection limit of 0.5 nmol/L. Four (19.0%) SalFDex samples in the hypercortisolism group and 68 (87.2%) SalFDex samples in the eucortisolism group

### Table 1. Baseline characteristics of study subjects

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>Hypercortisolism (n=21)</th>
<th>Eucortisolism (n=78)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58.9 ± 14.2</td>
<td>54.3 ± 14.3</td>
<td>0.320</td>
</tr>
<tr>
<td>Male:female</td>
<td>6:15</td>
<td>34:44</td>
<td></td>
</tr>
<tr>
<td>Obesity</td>
<td>9 (42.9%)</td>
<td>27 (34.6%)</td>
<td>0.488</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>5 (23.8%)</td>
<td>29 (37.2%)</td>
<td>0.255</td>
</tr>
<tr>
<td>Hypertension</td>
<td>17 (81.0%)</td>
<td>54 (69.2%)</td>
<td>0.292</td>
</tr>
<tr>
<td>Cushingoid features</td>
<td>10 (47.6%)</td>
<td>11 (14.1%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Proximal myopathy</td>
<td>4 (19.0%)</td>
<td>2 (2.6%)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* Data are shown as mean ± standard deviation, No. of patients, or No. (%) of patients, unless otherwise stated

### Table 2. Biochemical test results of study subjects

<table>
<thead>
<tr>
<th>Test</th>
<th>Median (range)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-overnight dexamethasone suppression serum cortisol (nmol/L)</td>
<td>206.0 (30-1081)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>24-Hour urinary free cortisol (nmol/d)</td>
<td>219.0 (80.0-7570)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Late-night salivary cortisol (nmol/L)</td>
<td>5.4 (1.7-41.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Late-night salivary cortisone (nmol/L)</td>
<td>30.0 (11.0-148.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Post-overnight dexamethasone suppression salivary cortisol (nmol/L)</td>
<td>2.0 (0.5-72)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Post-overnight dexamethasone suppression salivary cortisone (nmol/L)</td>
<td>25.0 (0.5-1100)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* 2 Sets of data which had inadequate late-night salivary samples for measurement were excluded
† 21.8% of subjects had levels below the detection limit (0.5 nmol/L)
‡ 19.0% of subjects had levels below the detection limit (0.5 nmol/L)
§ 87.2% of subjects had levels below the detection limit (0.5 nmol/L)
|| 1.3% of subjects had levels below the detection limit (0.5 nmol/L)
Salivary cortisone for Cushing’s syndrome

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had a level below the detection limit of 0.5 nmol/L. One (1.3%) SalEDex sample in the eucortisolism group had a level below the detection limit of 0.5 nmol/L.

The ROC analysis (Fig 1) and Table 3 reveal that the optimal cut-off for SalE_LN was 13.50 nmol/L. Setting the specificity at a level of 95%, the cut-off for SalE_LN would be 20.50 nmol/L.

After 1-mg overnight dexamethasone suppression, the optimal cut-off for SalF_Dex was 0.85 nmol/L (Table 3). Nonetheless, these values should be interpreted with caution, since many SalF_Dex values in both the eucortisolism (87.2%) and hypercortisolism (19.0%) groups were below the detection limit of the LC-MS/MS assay and were consequently presumed to be equivalent to the lowest detection limit of 0.5 nmol/L.

After 1-mg overnight dexamethasone suppression, the optimal cut-off for SalE_Dex was 7.45 nmol/L. Setting the sensitivity at a level of 95%, the cut-off for SalE_Dex would be 3.25 nmol/L (Table 3).

The correlation between 0900h serum cortisol and salivary cortisol was 0.81 (P<0.01); and that between 0900h serum cortisol and salivary cortisone was 0.88 (P<0.01). The correlation between SerF_Dex and SalF_Dex was 0.90 (P<0.01); that between SerF_Dex and SalE_Dex was 0.94 (P<0.01) [Fig 2].

Discussion

All investigators who study Cushing’s syndrome are confronted with the conundrum of accurately diagnosing or excluding the condition with no gold standard test.1 In our study, in addition to the histological criterion, we considered it appropriate to include a set of biochemical criteria in which subjects with two positive results among the three commonly used tests—namely the SerF_Dex, the UFC, and the SalF_LN—were considered to have hypercortisolism. This is in agreement with the Endocrine Society Clinical Practice Guideline17 that recommends performing one or two other tests if one of these is abnormal; and if results from two different tests are concordant, to proceed with investigations to establish the cause of Cushing’s syndrome. One well-recognised contentious point in the interpretation of the SerF_Dex is the optimal cut-off: <140 nmol/L is a widely cited normal response, but can lead to

<table>
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<tr>
<th>TABLE 3. Performance characteristics of late-night salivary cortisone, and post-1 mg overnight dexamethasone suppression salivary cortisol and cortisone</th>
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<tr>
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<tr>
<td>Late-night salivary cortisone</td>
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<tr>
<td>Post-overnight dexamethasone suppression salivary cortisol</td>
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<tr>
<td>Post-overnight dexamethasone suppression salivary cortisone</td>
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<tr>
<td>Post-overnight dexamethasone suppression salivary cortisone</td>
</tr>
</tbody>
</table>

Abbreviations AUC = area under the curve; LC-MS/MS = liquid chromatography–tandem mass spectrometry; SD = standard deviation

* Data to be interpreted with caution since many values are below the detection limit of the LC-MS/MS assay and taken to be equivalent to the lowest detection limit of 0.5 nmol/L

† The optimal cut-off is chosen where the Youden’s index, ie, “sensitivity + specificity-1”, is maximal
false-negative results in up to 15% of subjects with Cushing’s syndrome.\textsuperscript{18,19} The more stringent cut-off of $<50$ nmol/L sacrifices specificity for sensitivity.\textsuperscript{20,21} In this study, we adopted a double cut-off as proposed by the European Society of Endocrinology Clinical Practice Guideline in collaboration with the European Network for the Study of Adrenal Tumors\textsuperscript{15}; the rationale being that a more sensitive cut-off should be employed in those with a higher pretest probability of Cushing’s syndrome, such as the presence of an adrenal adenoma on imaging studies.\textsuperscript{22} A more specific cut-off can be employed in general to avoid over-diagnosis.

The loss of circadian rhythm with absence of a late-night cortisol nadir is a well-established feature of Cushing’s syndrome. Midnight serum cortisol is, however, difficult to obtain. When SalF\textsubscript{LN} was shown to correlate well with serum cortisol levels, with sensitivity of 92%-100% and specificity of 93%-100% for the diagnosis of Cushing’s syndrome,\textsuperscript{17} it rapidly became one of the most popular tests in investigating endogenous hypercortisolism. In view of the theoretical advantages of salivary cortisone, we also attempted to explore the performance characteristics of SalELN. Our data showed that it had a good sensitivity of 94.7% and a specificity of 87.2% at the cut-off of 13.50 nmol/L, as measured by LC-MS/MS.

We could not compare the utility of SalF\textsubscript{LN} with cortisone in this study, since SalF\textsubscript{LN} was one of the criteria applied to define Cushing’s syndrome. Simultaneous measurement of salivary cortisol and

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image}
\caption{Correlation plots between 0900h serum cortisol versus (a) salivary cortisol and (b) salivary cortisone; and between post-dexamethasone serum cortisol versus (c) salivary cortisol and (d) salivary cortisone.}
\begin{itemize}
\item (a) Pearson correlation coefficient = 0.81 (P<0.01)
\item (b) Pearson correlation coefficient = 0.86 (P<0.01)
\item (c) Pearson correlation coefficient = 0.90 (P<0.01)
\item (d) Pearson correlation coefficient = 0.94 (P<0.01)
\end{itemize}
\textbf{Abbreviation:} ONDST = overnight dexamethasone suppression test
\end{figure}
salivary cortisone can nonetheless alert clinicians to certain caveats encountered when measuring salivary cortisol alone. When the salivary cortisol-to-cortisone ratio is exceptionally high, direct contamination of the oral sample by topical or oral hydrocortisone must be excluded. Ingestion of glycyrrhetinic acid (eg in licorice, carbenoxolone), which competitively inhibits 11β-HSD2, or rare cases of genetic 11β-HSD2 defect, can also lead to the same anomaly.

A number of other investigators have explored the utility of SalF₉Dex in the diagnosis of Cushing’s syndrome. Apart from its convenience, salivary values are not affected by conditions that affect corticosteroid-binding globulin (CBG) or albumin levels, such as acute and chronic illness, pregnancy or oestrogen treatment, or genetic variants of CBG. A sensitivity varying between 97% and 100% and a specificity between 77% and 100% have been variously reported, with cut-off level varying between 1.7 nmol/L and 2 nmol/L.⁶⁻⁹ In the current study, we found that the sensitivity of SalF₉Dex was only 76.2% at the optimal cut-off of 0.85 nmol/L. Although this discrepancy with other studies might be due to a number of factors, such as the means of defining normal ranges and the criteria for diagnosing Cushing’s syndrome, one important factor that is evident from our data is the method used for assaying salivary cortisol: some used electrochemiluminescence assay;⁹ others used radioimmunoassay;⁶⁻⁸; but we measured SalF₉Dex with LC-MS/MS.¹² Unlike immunoassays, LC-MS/MS measurement of analytes is more specific, with less cross-reactivity among different cortisol precursors and metabolites.²¹ The concentration of SalF₉Dex was very low: 19% of our patients with hypercortisolism and 87% of those with eu-cortisolism had SalF₉Dex below the detection limit of 0.5 nmol/L, leading to uncertainty in establishing the cut-off, since all those with results of <0.5 nmol/L could only be considered to have salivary cortisol level equal to 0.5 nmol/L in the analysis. Immunoassays, by measuring other cortisol precursors or metabolites in varying degrees in addition, could have bypassed this problem. Other studies have also reported that SalF₉LN has poorer diagnostic performance characteristics if measured by LC-MS/MS, in comparison with the less-specific immunoassays such as chemiluminescent assays or radioimmunoassays.²⁴⁻²⁵

Nevertheless, LC-MS/MS is analytically more superior and is expected to become the steroid assay of choice in the future.²⁶ Values generated by studies using LC-MS/MS have greater inter-centre and long-term generalisability in view of the lack of assay-specific steroid cross-reactivity. Adoption of cut-offs generated by studies in which salivary cortisol was assayed using antibody-based methods into clinical practice is known to be problematic.²⁷ Individual centres are often advised to generate their own references and cut-offs although this is often not feasible. In a meta-analysis on the use of SalF₉₄¹ for investigation of Cushing’s syndrome,²⁵ the recommended cut-offs varied widely, from 3.59 to 15.17 nmol/L. To overcome the problem of lower performance characteristics due to low levels of salivary cortisol, instead of going for immunoassays, a better solution may be to measure salivary cortisone that is present in a much higher concentration than salivary cortisol. At a serum cortisol below 74 nmol/L, Debono et al²⁸ showed that salivary cortisol could become undetectable by LC-MS/MS, while salivary cortisone was always detected. Similarly, our data showed that after dexamethasone suppression, when the salivary cortisol became too low to be measured with LC-MS/MS in many subjects, salivary cortisone could still be measured in all but one subject in the eu-cortisolism group.

Between salivary cortisol and salivary cortisone, this study showed that salivary cortisone would be the preferred test because it is present at a higher concentration in the saliva; and at comparable specificity levels, SalE₉Dex appears to have better accuracy (as reflected by the higher AUC of the ROC curves), sensitivity, and negative LR than SalF₉Dex.²³ Apart from the optimal cut-off, clinically it is often useful to have two cut-offs, one for ruling in a diagnosis (high specificity) and another one for excluding a diagnosis (high sensitivity), depending on clinician preference. If we arbitrarily define an acceptable and useful cut-off as having a 95% level of either sensitivity or specificity, the two useful cut-offs for SalE₉Dex as derived from our study were 13.50 and 20.50 nmol/L, respectively; those for SalF₉Dex were 3.25 nmol/L and 7.45 nmol/L, respectively.

Traditionally, in the algorithm for the workup for Cushing’s syndrome, late-night levels (serum or salivary cortisol) have been used for screening (excluding Cushing’s syndrome), whereas the post-dexamethasone level (serum cortisol) has been used for diagnosis (ruling in Cushing’s syndrome). When used as such, the cut-off of 13.50 nmol/L can be used for SalE₉LN; whereas 7.45 nmol/L can be used for SalE₉Dex. For the time being, SalE₉Dex data can supplement serum cortisol measurement as a confirmatory test when concordant, or alert the clinician to the potential pitfalls with serum cortisol (eg variations in CBG levels) when discordant. With more experience, SalE₉Dex may even ultimately replace the need to measure serum cortisol.

The strength of this study lies in the rigour with which a pre-specified protocol was adhered to. A high success rate of sample collection was achieved, with little missing data. Insufficient salivary volume collected in the Salivette tubes was the most common reason for unsuccessful salivary collection, because LC-MS/MS requires a larger
saliva volume (100-250 μL) than immunoassay (40-50 μL). Two thirds of our study subjects were referred either because of an adrenal incidentaloma or common clinical conditions such as diabetes mellitus and hypertension, and had no clinical features of Cushing’s syndrome. This population was quite representative of cases referred to an endocrine centre for workup of Cushing’s syndrome.

A notable limitation of this study is the small number of subjects with hypercortisolism. The cut-off for the SerFDex was adopted from the literature rather than from data derived from our own healthy volunteers. The Endocrine Society Clinical Practice Guideline recommended two separate measurements of SalF LN or UFC. Only one likely be in a phase of normal cortisol secretion even than two out of the relatively sensitive tests were episodic hypercortisolism, we assumed that if less than two out of the relatively sensitive tests were positive at the time of sampling, the subjects would likely be in a phase of normal cortisol secretion even if they had episodic Cushing’s syndrome.

**Conclusion**

Our study showed that salivary cortisol can become the analyze of choice for investigating Cushing’s syndrome in the era of LC-MS/MS. Our data suggest using 13.50 nmol/L for SalELN, and either 7.45 nmol/L (more specific) or 3.25 nmol/L (more sensitive) for SalEDex as cut-offs.

**Declaration**

All authors have disclosed no conflicts of interest.

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