Prospective Evaluation of Late-Night Salivary Cortisol and Cortisone by EIA and LC-MS/MS in Suspected Cushing Syndrome

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Context. Late-night salivary cortisol (LNSC) measured by enzyme immunoassay (EIA-F) is a first-line screening test for Cushing syndrome (CS) with a reported sensitivity and specificity of >90%. However, liquid chromatography-tandem mass spectrometry, validated to measure salivary cortisol (LCMS-F) and cortisone (LCMS-E), has been proposed to be superior diagnostically.

Objective, Setting, and Main Outcome Measures. Prospectively evaluate the diagnostic performance of EIA-F, LCMS-F, and LCMS-E in 1453 consecutive late-night saliva samples from 705 patients with suspected CS.

Design. Patients grouped by the presence or absence of at least one elevated salivary steroid result and then subdivided by diagnosis.

Results. We identified 283 patients with at least one elevated salivary result; 45 had an established diagnosis of neoplastic hypercortisolism (CS) for which EIA-F had a very high sensitivity (97.5%). LCMS-F and LCMS-E had lower sensitivity but higher specificity than EIA-F. EIA-F had poor sensitivity (31.3%) for adrenocorticotropic hormone (ACTH)-independent CS (5 patients with at least 1 and 11 without any elevated salivary result). In patients with Cushing disease (CD), most nonelevated LCMS-F results were in patients with persistent/recurrent CD; their EIA-F levels were lower than in patients with newly diagnosed CD.

Conclusions. Since the majority of patients with ≥1 elevated late-night salivary cortisol or cortisone result did not have CS, a single elevated level has poor sensitivity and positive predictive value. LNSC measured by EIA is a sensitive test for ACTH-dependent Cushing syndrome but not for ACTH-independent CS. We suggest that neither LCMS-F nor LCMS-E improves the sensitivity of late-night EIA-F for CS.

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Key Words: Cushing disease, ectopic ACTH, adrenal Cushing syndrome, diagnosis, assay performance

Abbreviations: ACTH, adrenocorticotropic hormone; BMI, body mass index; CD, Cushing disease; CS, Cushing syndrome; CV, coefficient of variation; DST, dexamethasone suppression test; EIA, enzyme immunoassay; EIA-F, cortisol measurement by enzyme immunoassay; LCMS-E, cortisone measurement by LC-MS/MS; LCMS-F, cortisol measurement by LC-MS/MS; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LNSC, late-night salivary cortisol.
The diagnosis of endogenous neoplastic hypercortisolism—Cushing syndrome (CS)—is one of the most challenging in clinical medicine. Neoplastic hypercortisolism is often responsible for cardiometabolic problems, including obesity, diabetes, hypertension, and low bone density, as well as significant neurocognitive and neuropsychiatric challenges [1-16]. Patients with CS have an increased standard mortality ratio that may be reduced by effective therapy [17-22]. The therapeutic landscape with surgery, radiotherapy, and pharmacotherapy has improved dramatically over the past 30 years for CS and has resulted in better patient outcomes [23]. Accordingly, the early diagnosis of CS with sensitive diagnostic tests is important to establish a timely diagnosis.

Late-night salivary cortisol (LNSC) measured by immunoassay is a useful and simple means to screen patients for suspected hypercortisolism and has been recommended by expert guidelines as an important first-line diagnostic test [24, 25]. A commonly used cortisol enzyme immunoassay (EIA-F) is inexpensive, Food and Drug Administration (FDA)-cleared, available internationally, and readily performed in clinical laboratories [26, 27]. Furthermore, high-throughput platform cortisol immunoassay systems are widely available at reference laboratories around the world, although there are significant differences in their performance and reference ranges [28, 29].

It has been proposed that measuring late-night salivary cortisol and cortisone by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (LCMS-F and LCMS-E) may provide improved diagnostic characteristics compared to EIA-F due to (a) its better analytical specificity and (b) the fact that salivary cortisone is typically higher than salivary cortisol due to the expression of 11-beta-hydroxysteroid dehydrogenase (11βHSD) type 2 in the salivary gland, possibly amplifying the signal and making it more useful diagnostically [28-31]. On the other hand, measuring other cortisol metabolites by the typically lower analytically specific immunoassays may improve diagnostic sensitivity for CS compared to the more analytically specific LCMS-F [32-35]. In addition, a very high LCMS-F to LCMS-E ratio in the presence of very high measured salivary cortisol by EIA-F or LCMS-F is useful as an indicator of contamination of the salivary sample with topical or oral hydrocortisone (authentic cortisol) [36].

In the current study, we prospectively evaluated consecutive salivary samples referred to Wisconsin Diagnostic Laboratories in a calendar year. We identified all samples with at least one elevated salivary steroid result compared with a randomly selected subset of those with no elevated results, extracted clinical data from the medical records, and correlated them with the late-night EIA-F, LCMS-F, and LCMS-E results. All patients’ records were evaluated for the final diagnosis of biochemical and/or clinical CS. We addressed the hypothesis that the measurement of LCMS-F and/or LCMS-E may improve on the known excellent sensitivity of late-night EIA-F measurement for the diagnosis of CS.

Methods

The study was approved by the Medical College of Wisconsin/Froedtert Hospital Institutional Review Board with a Material Transfer Agreement with Aurora Health Care/Aurora Research Institute. All samples transferred were de-identified.

Study design

All late-night salivary samples ordered by any clinician as part of clinical care were processed through Wisconsin Diagnostic Laboratories and collected for 1 calendar year (January 1, 2019 through December 31, 2019). A total of 1453 salivary samples from 705 patients were collected during this time using a standard Salivette (Sarstedt, Newton, NC). In addition to the standard FDA-cleared EIA-F performed on these samples [27], each sample underwent additional testing to obtain LCMS-F and LCMS-E concentrations [26]. The 1453 samples were first subdivided into 2 groups (Fig. 1): samples with all normal late-night salivary steroid data and samples with at least one abnormal value among the 3 salivary analyses.
Abnormal late-night salivary results were defined as EIA-F ≥3.3 nmol/L; LCMS-F ≥2.8 nmol/L, and LCMS-E ≥8.7 nmol/L based on our previously published contemporaneous normative data using the same analytic methods in samples collected in the same manner [26]. We identified 501 samples from a total of 283 patients with at least one abnormal salivary result and 952 samples in 422 patients without increases in any salivary result. In patients with more than one late-night salivary sample, we chose the sample to include in the numerical analyses that had the highest EIA-F, LCMS-F, and/or LCMS-E result. Medical records of these 422 patients were evaluated to determine whether they had any other biochemical evidence of endogenous hypercortisolism. Out of those with no evidence of biochemical hypercortisolism, 121 patients were randomly selected for an in-depth medical record review and served as a control group. An in-depth medical record review was performed on all 283 patients with any abnormal salivary steroid testing, as well as the 121 patients randomly selected with all normal late-night salivary data. We also identified whether patients had any prior relevant endocrine imaging or surgery (pituitary, adrenal). Those patients with established CS were identified as having pituitary (CD), adrenal, or ectopic CS. Those with CD were further subdivided into those with de novo disease and those who had either persistent or recurrent CD after pituitary surgery.

**Salivary assays**

Salivary cortisol was measured by EIA using an FDA-cleared method (Salimetrics, State College, PA) as described in detail previously [26, 37]. The lower detection limit is 0.3 nmol/L. Samples with LNSC-EIA results >80 nmol/L were assayed after a dilution of 1:20. The intraassay coefficient of variation (CV) is 5.2% at 3.1 nmol/L (n = 10) and 2.6% at 10.4 nmol/L (n = 10). Interassay (total) imprecision (CV) is 11% at 2.8 nmol/L (n = 10), 11% at 10.1 nmol/L (n = 10), and 6.9% at 25.0 nmol/L (n = 10). Relevant endogenous steroid cross-reactivities are cortisol (0.13%), 11-deoxycortisol (0.16%), and corticosterone (0.21%).

Salivary cortisol and cortisone were measured by LC-MS/MS as described by us in detail previously [26]. The functional sensitivity, set at a threshold CV of 10%, was 0.053 (SD, 0.004) nmol/L for cortisol and 0.053 (SD, 0.002) nmol/L for cortisone. Therefore, the analytic range of the LC-MS/MS method for cortisol and cortisone was conservatively set at 0.1 to 89.4 nmol/L. The intraassay variability (N = 10) for cortisol is 7.1% at 1.4 nmol/L, 3.1% at 5.8 nmol/L, and 2.7% at 10.3 nmol/L and for cortisone is 4.8% at 4.2 nmol/L, 3.2% at 23.3 nmol/L, and 2.9% at 31.9 nmol/L. The interassay variability (N = 20) for cortisol is
11.1% at 1.3 nmol/L, 6.5% at 4.6 nmol/L, and for cortisone is 8.5% at 3.6 nmol/L and 5.8% at 20.2 nmol/L.

Potential blood contamination of saliva was evaluated in a subset of samples without and with modest increases in cortisol by EIA with a ratio of cortisol to cortisone greater or less than a cutoff of 1 [36]. To do this, salivary transferrin was measured by enzyme immunoassay (Salimetrics, State College, PA) as validated previously [38, 39]. The sensitivity of this assay is 0.08 mg/dL. The intra-(N = 12) and inter-assay (N = 10) variabilities are 4.9% to 10.2% and 7.1% to 7.2%, respectively.

**Statistical analysis**

Continuous data were evaluated by Mann-Whitney Rank Sum test or Kruskal-Wallis Analysis of Variance on Ranks (with Dunn’s all pairwise multiple comparisons) and presented as median [interquartile range] or t-test and presented as mean [standard deviation (SD)] (Sigmaplot 12.5, Systat Software Inc., San Jose, CA). Categorical data were evaluated by chi-square. P < 0.05 was considered significant. Salivary steroid vs salivary transferrin data were also evaluated by linear regression. Diagnostic performance of each salivary assay was evaluated using MEDCALC [40].

**Results**

We collected 1453 late-night salivary samples from 705 patients (approximately 2 samples per patient) (Fig. 1). Of those, 501 samples from 283 patients had at least one salivary steroid result above the established cutoff for that analytic method. The remaining 952 samples from 422 patients had no elevated salivary cortisol result. Of those, 391 patients did not have any clinical or biochemical evidence of neoplastic hypercortisolism (active Cushing syndrome). From that group of 391 patients, we randomly selected 121 patients and extracted detailed results from the medical record to use as a control group. Of the remaining 31 patients without an increase in any salivary results, 20 could not be evaluated because of lack of access to their extramural medical records and 11 were identified as having adrenal CS. In these 11 patients, post–low-dose dexamethasone suppression test (DST) serum cortisol was 3.4 [1.9-7.2] µg/dL. These post-DST serum cortisol results were not different from the patients with adrenal CS (8.2 [2.8-15.7] µg/dL; P = 0.169) who had at least one elevated late-night salivary result. Of the 283 patients with at least one elevated salivary cortisol result, we identified 45 patients with proven CS (Fig. 1). Of these, 40 had ACTH-dependent CS (35 with Cushing disease and 5 with ectopic ACTH). The remaining 5 patients had adrenal (ACTH-independent) CS. Of these 5 patients with adrenal CS, all had elevated EIA-F, 4 had elevated LCMS-F, and 3 had elevated LCMS-E. For purposes of calculating assay performance, the 5 patients with adrenal CS identified by at least one elevated salivary steroid measurement were combined with the 11 patients with adrenal CS who had no elevated salivary steroid result but all had an abnormal DST (Fig. 1).

Pertinent patient data, divided into no increased salivary results and at least one increased salivary result, are shown in Table 1. The group with adrenal CS without any elevated salivary cortisol results were older compared with the other 4 groups. The patients with ectopic ACTH had significantly lower body mass index (BMI). There was no difference in sex distribution between the groups, with all groups having more female than male patients. There was no difference between any salivary cortisol result between the control group (no evidence of Cushing syndrome) compared to the patients with adrenal CS without elevated salivary steroids. EIA-F, LCMS-F, and LCMS-E were greater in all patients with at least one elevated salivary result compared to normal patients or patients with adrenal CS without any elevated late-night salivary results. Furthermore, patients with at least one elevated salivary cortisol result had a significantly higher ratio of salivary cortisol to cortisone.
Assay performance in patients with ACTH-dependent CS (Cushing disease and ectopic ACTH) is shown in Table 2. EIA-F had the highest sensitivity but the lowest specificity, whereas LCMS-F had the lowest sensitivity and highest specificity. The positive predictive value of all of the tests was low, but the negative predictive value was very high.

Assay performance in patients with ACTH-independent CS (5 patients with increased salivary steroids and 11 without increased salivary steroids) are shown in Table 3. Because of the large number of nonelevated salivary cortisol results, the sensitivity was low, whereas the specificity was similar to patients with ACTH-dependent CS shown in Table 2. As a result, the positive predictive value of any salivary test was very low in patients with ACTH-independent Cushing syndrome.

Table 4 shows the distribution of salivary results in patients with at least one elevated salivary steroid result. The only false-negative EIA-F result was in a patient with Cushing disease 9 years after pituitary surgery (2.4 nmol/L [cutoff 3.3 nmol/L]). This patient had a slightly elevated LCMS-E (9.3 nmol/L [cutoff 8.7 nmol/L]) and a normal LCMS-F (1.4 nmol/L [cutoff 2.8 nmol/L]). One patient with ectopic ACTH had an LCMS-F of 2.6 nmol/L that was just below the cutoff of 2.8 nmol/L, while LCMS-E and EIA-F

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**Table 1. Patient Data**

|                      | No increased salivary results | At least one increased salivary result |
|----------------------|-------------------------------|---------------------------------------|
|                      | Normal Patients               | Adrenal Cushing Syndrome              | Cushing Disease | Ectopic ACTH |
|                      | (N = 121)                     | (N = 11)                              | (N = 35)       | (N = 5)      | (N = 5)      |
| Age, years           | 42 (34-59)                    | 60 (52-62)a                           | 50 (35-63)     | 50 (40-60)   | 46 (38-70)   |
| BMI, kg/m²           | 34.3 (28.6-39.8)              | 30.5 (26.0-39.0)                      | 34.3 (28.3-43.2) | 24.2 (22.0-29.8)b | 38.2 (30.0-44.2) |
| Female/Male          | 104/17                        | 8/3                                   | 25/10          | 5/0          |
| EIA-F, nmol/L        | 0.9 (0.7-1.4)                 | 1.7 (0.9-1.8)                         | 7.4 (4.5-11.5)c | 25.2 (5.9-176.0)c | 13.5 (6.7-48.6)c |
| LCMS-F, nmol/L       | 0.5 (0.3-0.8)                 | 0.7 (0.6-1.0)                         | 3.5 (2.2-7.5)c | 17.5 (3.1-122.8)c | 8.0 (3.5-42.5)c |
| LCMS-E, nmol/L       | 3.7 (2.3-4.9)                 | 4.5 (3.7-5.4)                         | 18.5 (11.9-27.0)c | 52.8 (13.1-200.8)c | 25.8 (7.7-50.5)c |
| LCMS-F/E ratio       | 0.15 (0.11-0.19)              | 0.17 (0.13-0.20)                      | 0.20 (0.16-0.32)c | 0.33 (0.24-0.65)c | 0.34 (0.20-1.29)c |

Except for Female/Male ratio; data are median (interquartile range). BMI: N = 119 for Normal Patients. 
Abbreviations: BMI, body mass index; E, cortisone; F, cortisol.

*aDifferent from all other groups by Mann-Whitney rank sum test (P = 0.008-0.032). bDifferent from normal by Mann-Whitney rank sum test (P = 0.006). cDifferent from Normal by Kruskal-Wallis 1-way analysis of variance on ranks (P < 0.001) and all pairwise multiple comparison procedure (Dunn’s method). Female/Male data analyzed by Chi-square test.

**Table 2. Performance of Different Salivary Assays in Patients (N = 40) with ACTH-Dependent Cushing Syndrome (Cushing Disease [N = 35]; Ectopic ACTH [N = 5]) Compared to Patients without Cushing Syndrome Proven (N = 629). Parentheses Denote 95% Confidence Limits**

| Assay Cutoff | ACTH-dependent Cushing Syndrome | Positive Predictive Value (PPV) | Negative Predictive Value (NPV) |
|--------------|---------------------------------|---------------------------------|---------------------------------|
| +/−          | Yes                             | No                              | Sensitivity                      |
| EIA-F        | 39/1                            | 193/436                         | 97.5 (86.8-99.9)                 |
| LCMS-F       | 27/13                           | 97/532                          | 67.5 (50.9-82.4)                 |
| LCMS-E       | 37/3                            | 150/479                         | 92.5 (72.6-98.4)                 |
|              |                                 |                                  | 69.3 (65.6-72.9)                 |
|              |                                 |                                  | 84.6 (81.5-87.3)                 |
|              |                                 |                                  | 76.2 (72.6-79.4)                 |

Sensitivity, specificity, PPV, and NPV are in percentages.
were both increased (10.9 nmol/L and 6.9 nmol/L, respectively). One patient with adrenal Cushing had LCMS-F of 1.7 nmol/L and an LCMS-E of 8.4 (both below cutoffs), whereas the EIA-F was 3.7 nmol/L (just above the cutoff of 3.3 nmol/L). In the 238 patients with at least one elevated result, most had false positive EIA-F, whereas the fewest had false positive LCMS-F.

We then performed a detailed evaluation of the 35 patients with ACTH-dependent pituitary Cushing syndrome (CD). The salivary results from these patients from Table 1 are plotted in Fig. 2 to better visualize the data. The analytic method with the least overlap of outliers (solid circles) was EIA-F whereas the overlap for LCMS-F and LCMS-E was greater. There was considerable overlap of salivary cortisol F/E ratio between the groups. When considering the frequency of false negative results in patients with known CD (salivary steroid below the established assay cutoffs in healthy subjects [26]), it is clear that LCMS-F had the most (12 of 35; 34%) compared to EIA-F (1 of 35; 3%) and LCMS-E (3 of 35; 9%) (Fig. 3). EIA-F and LCMS-E were not different from each other in this regard.

We parsed the CD data into those patients with de novo disease (n = 12) compared with those with biochemical recurrence or persistence after pituitary surgery (n = 23) (Table 5). BMI tended to be lower, but was not significantly so, in patients after pituitary surgery with recurrence or persistence of disease. Not surprisingly, EIA-F, LCMS-F, and LCMS-E were lower after pituitary surgery, although they were still increased compared with control subjects, shown in Table 1. It is clear from Fig. 4 that most of the “false negatives” from Fig. 3 were in patients with postsurgical persistence or recurrence reflecting their milder degree of hypercortisolism and, therefore, closeness to the assay cutoffs.

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**Table 3. Performance of Different Salivary Assays in Patients (N = 16) With ACTH-Independent (adrenal) Cushing Syndrome Compared With Patients Without Proven Cushing Syndrome (N = 629). Parentheses Denote 95% Confidence Limits**

| Assay Cutoff       | + Above | - Below | Yes     | No     | Sensitivity | Specificity | Positive Predictive Value (PPV) | Negative Predictive Value (NPV) |
|--------------------|---------|---------|---------|--------|-------------|------------|---------------------------------|---------------------------------|
| EIA-F +/-          | 5/11    | 193/436 | 31.3    | 69.3   | (11.0-58.7) | (65.6-72.9) | 2.5 (1.2-5.1)                  | 97.5 (96.6-98.2)                |
| LCMS-F +/-         | 4/12    | 97/532  | 25.0    | 84.6   | (7.3-52.5)  | (81.5-87.3) | 4.0 (1.7-9.0)                  | 97.8 (97.1-98.3)                |
| LCMS-E +/-         | 3/13    | 150/479 | 18.8    | 76.2   | (4.1-45.7)  | (72.6-79.4) | 2.0 (0.7-5.3)                  | 97.4 (96.7-97.9)                |

Sensitivity, specificity, PPV, and NPV are in percentages.

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**Table 4. Breakdown of Assay Results in Patients With at Least 1 Abnormal Salivary Result.**

| Abnormal Result | Total (45) | CD (35) | Ectopic (5) | Adrenal (5) | CS not proven (238) |
|-----------------|------------|---------|-------------|-------------|---------------------|
| EIA-F           | 44 (98%)   | 34 (97%)| 5 (100%)    | 5 (100%)    | 193 (81%)           |
| LCMS-F          | 31 (69%)   | 23 (66%)| 4 (80%)     | 4 (80%)     | 97 (41%)            |
| LCMS-E          | 39 (87%)   | 32 (91%)| 5 (100%)    | 3 (60%)     | 150 (63%)           |

N values and percentages of patients in that column shown in parentheses.
Abbreviations: CD, Cushing disease; CS, Cushing syndrome; EIA-F, cortisol measurement by enzyme immunoassay; LCMS-E, cortisone measurement by LC-MS/MS; LCMS-F, cortisol measurement by LC-MS/MS.
Figure 2. Late-night salivary enzyme immunoassay cortisol (EIA-Cortisol), liquid chromatography-tandem mass spectrometry (LCMS) cortisol and cortisone, and the ratio of LCMS cortisol to cortisone in 35 patients with Cushing disease compared with the 121 patients randomly chosen with all normal salivary steroid results and without the diagnosis of Cushing syndrome of any type. Horizontal line is the median; box indicates 25th to 75th percentile, whisker indicates 10th and 90th percentiles, and outliers are indicated by circles. P values are from Mann-Whitney Rank Sum tests.

Figure 3. Number of Cushing disease patients (N = 35) with late-night EIA-Cortisol and LC-MS/MS cortisol and cortisone equal to or below (Normal) or above (Abnormal) the reference range for that assay [26]. P values are from chi-square analysis.
The measurement of LCMS-F and LCMS-E allows the evaluation of F to E ratio. We did not find this ratio helpful diagnostically. Although the ratio is typically <0.5 due to the conversion of salivary F to E by salivary 11βHSD-type 2 [30, 31, 36, 41], samples with an increased F to E ratio are occasionally found. When we obtain very high EIA-F or LCMS-F results (>100 nmol/L) and the LCMS-F to LCMS-E ratio is very high (>30), it is typically due to contamination with topical hydrocortisone (authentic cortisol) [36]. When we evaluated the current data set, we found samples from 20 patients with a mild increase in EIA-F and F to E ratios >1 and no evidence of topical or oral hydrocortisone use in the medical record. We thought this might be due to blood contamination not visible when the saliva samples were visually screened. Therefore, we measured salivary transferrin concentrations in samples from these 20 patients compared to 19 matched patients selected with the same increase in EIA-F and normal F to E ratios. Table 6 demonstrates that there was no difference in transferrin concentrations between the 2 groups. Furthermore, we demonstrate in Fig. 5 that there was no correlation between salivary EIA-F, LCMS-F, LCMS-E, or the ratio of F to E, indicating a lack of concurrence with transferrin.

Table 5. De Novo CD vs Patients With Postoperative Recurrence/Persistence of CD

|                      | De Novo CD | Recurrent/Persistent CD |
|----------------------|------------|-------------------------|
|                      | Median (IQR) | Median (IQR) | P values |
|                      | (N = 12)    | (N = 23)               |          |
| Age, years           | 47 (33-61)  | 51 (38-68)             | 0.375    |
| BMI, kg/m²           | 38.5 (32.5-46.1) | 32.0 (24.3-38.5) | 0.063    |
| Female/Male          | 7/5         | 18/5                   | 0.258    |
| EIA-F, nmol/L        | 12.2 (6.5-22.8) | 6.5 (4.1-8.6) | 0.010    |
| LCMS-F, nmol/L       | 7.6 (3.5-10.3) | 3.3 (2.0-5.0) | 0.017    |
| LCMS-E, nmol/L       | 27.6 (19.7-38.9) | 14.2 (9.3-24.2) | 0.002    |
| LCMS-F/E ratio       | 0.21 (0.14-0.30) | 0.19 (0.17-0.40) | 1.000    |

Data analyzed by Mann-Whitney Rank Sum test and Chi-Square Test (Female/Male).
Abbreviations: BMI, body mass index; CD, Cushing disease; EIA-F, cortisol measurement by enzyme immunoassay; IQR, interquartile range; LCMS-E, cortisone measurement by LC-MS/MS; LCMS-F, cortisol measurement by LC-MS/MS.

Figure 4. Number of Cushing disease patients with de novo Cushing disease (N = 12) and patients with recurrent/persistent Cushing disease (N = 23) with late-night EIA-Cortisol and LC-MS/MS cortisol and cortisone equal to or below (Normal) or above (Abnormal) the reference range for that assay [26]. P values indicate comparison of LC-MS/MS cortisol vs other 2 analytes.
Discussion

Late-night salivary cortisol (LNSC) is a reliable, simple, and cost-effective first-line test for the diagnosis of endogenous neoplastic hypercortisolism (CS) [15, 16, 34, 35, 42-52]. We and others have demonstrated that, using relatively nonspecific immunoassays, the sensitivity and specificity of this test exceeds 90% and is as high as 95% to 97% [47, 53]. However, these previous studies demonstrating high specificity were generally from referral centers and did not consider the modern liberal use of LNSC as a screening test for CS amongst primary care physicians resulting from the publication of practice guidelines [54].

It has been suggested that the measurement of salivary cortisol (F) and cortisone (E) by LC-MS/MS may improve the performance of the test because of the high analytic specificity of this structural analytic methodology [28-31, 55-60]. Conversely, we have proposed that the detection of cortisol metabolites by relatively nonspecific immunoassays may amplify

| Salivary Transferrin (mg/dL; median [25%-75%]), Salivary Steroids (nmol/L; mean [SD]), and Salivary Cortisol to Cortisone Ratio (median [25%-75%]) partitioned by cortisol/cortisone ratio <1 vs ≥1 and modest increases in EIA cortisol. Samples chosen to match EIA-Cortisol between columns |
|---------------------------------------------------------------|
| **<1**                                                                 |
| Transferrin 0.50 [0.30-1.2]                                   |
| EIA-Cortisol 10.2 [5.2]                                        |
| LCMS-Cortisol 5.2 [2.8]                                        |
| LCMS-Cortisone 20.0 [11.5]                                     |
| Cortisol/Cortisone ratio 0.24 [0.19-0.34]                       |
| **≥1**                                                                 |
| (n = 20)                                                      |
| Transferrin 0.75 [0.25-1.40]                                   |
| EIA-Cortisol 10.1 [6.8]                                        |
| LCMS-Cortisol 7.1 [5.2]                                        |
| LCMS-Cortisone 3.1 [2.4]                                       |
| Cortisol/Cortisone ratio 2.23 [1.64-3.18]                      |
| **P values**                                                   |
| (n = 19)                                                      |
| Transferrin 0.725                                               |
| EIA-Cortisol 0.976                                             |
| LCMS-Cortisol 0.172                                            |
| LCMS-Cortisone <0.001                                          |
| Cortisol/Cortisone ratio <0.002                                 |

Between-column statistics: Mann-Whitney Rank Sum test for transferrin and F/E ratio; t-test for salivary steroid concentrations

**Figure 5.** Correlation of salivary steroid measurements vs salivary transferrin concentrations in 39 patients from Table 6. There were no significant correlations found.
the endogenous glucocorticoid signal and improve diagnostic sensitivity, albeit at lower specificity [34, 35].

The purpose of the current study was to prospectively evaluate a large number of late-night salivary cortisol samples (N = 1453) obtained from 705 patients with suspected CS. These tests were ordered by endocrinologists, endocrine surgeons, advanced practice providers, and primary care physicians making it more representative of the real world, rather than studies from only tertiary referral centers with a focus on CS [47, 53, 54]. Our approach, therefore, reduced ascertainment bias and gave a more realistic evaluation of the salivary tests.

Late-night EIA-F had the highest sensitivity for ACTH-dependent CS but the lowest specificity. This is fitting, considering the significant cross-reactivity of the EIA-F antibody with steroids other than cortisol [27]. The higher sensitivity of LCMS-E compared to LCMS-F for ACTH-dependent CS supports this concept in that salivary cortisone has a higher concentration in saliva, thus amplifying the endogenous glucocorticoid signal. Ultimately, EIA-F has a very high negative predictive value (99.8%) but a low positive predictive value (16.8%) for ACTH-dependent CS. This argues that complementary tests of adrenal function are required to establish the diagnosis but that a patient with a normal result is unlikely to have ACTH-dependent CS [61].

None of the salivary steroid methods provided an adequate sensitivity and positive predictive value for ACTH-independent (adrenal) CS considering that, in our study, 11 of 16 of these patients did not have any elevated salivary results. This is not surprising, since most studies have found that LNSC (regardless of assay method) has a poor sensitivity in patients with mild cortisol excess (formerly known as subclinical CS) [20, 56, 62-76]. Accordingly, other studies such as the overnight 1 mg dexamethasone suppression test (DST) should be the initial test in patients with adrenal nodular disease or adrenal incidentalomas [19, 56, 64, 65, 74].

Although EIA-F had better sensitivity than LCMS-F for the diagnosis of ACTH-dependent CS, many patients without neoplastic hypercortisolism had solitary elevations of EIA-F limiting its specificity and positive predictive value. There are many explanations for false positive LNSC for endogenous hypercortisolism that need to be considered. Pre-analytic errors, such as contamination of the sample with topical steroids is well appreciated and must always be considered in patients with an elevated EIA-F or LCMS-F and an increased LCMS-F/LCMS-E ratio [36]. In our study, although the ratio was not helpful diagnostically, it was higher in patients with endogenous hypercortisolism. We have previously shown that, even in healthy subjects, increased salivary cortisol is associated with an increase in the LCMS-F/LCMS-E ratio [26]. Some have speculated that an elevated F/E ratio can be due to blood contamination [39]. We showed that, once samples with visual blood were eliminated, salivary transferrin as an assessment of blood contamination did not account for samples with modest increases in EIA-F and higher LCMS-F to LCMS-E ratios.

Other causes of elevated LNSC in patients without neoplastic hypercortisolism include proximal stress, an abnormal sleep-wake cycle, inappropriate sampling time, aging, and smoking. Of course, many nonneoplastic forms of hypercortisolism, such as chronic kidney disease stage 5 and alcohol abuse disorder may also be associated with elevations of LNSC [61, 77]. Usage of different reference ranges may also cause confusion [28, 29]. Despite the many false positive EIA-F measurements, the near absence of any false negative results makes it a valuable screening test for the initial evaluation of patients suspected of ACTH-dependent CS.

It is increasingly appreciated that as many as 30% to 50% of CD patients will have either persistent or recurrent hypercortisolism after initial pituitary surgery [78-81]. Our data demonstrated that EIA-F had fewer false negative results than LCMS-F in the diagnosis of recurrent/persistent CD. Several studies have now shown that loss of the nadir of cortisol secretion late at night is the earliest detectable biochemical abnormality in patients with recurrent CD often preceding elevations of urine cortisol or an abnormal low-dose DST
Experts have now recommended an annual assessment of LNSC in order to establish an early diagnosis of recurrent CD [82, 84-86].

The diagnosis of new or recurrent CD requires careful evaluation of clinical findings with biochemical confirmation. Since sensitivity should be maximized in screening tests, the excellent sensitivity and negative predictive value of LNSC makes it a valuable screening tool. However, other clinical findings and biochemical tests, including DST, are needed to provide confirmation of CD [18, 24, 45, 87].

This single-center prospective study of late-night measurement of salivary cortisol (EIA-F or LCMS-F) and cortisone (LCMS-E) demonstrates that, considering all patients with neoplastic hypercortisolism, none of the methods of measurement have optimal specificity. Normal late-night cortisol or cortisone measurements provide excellent negative predictive value for ACTH-dependent CS (CD and ectopic ACTH); however, specificity is not optimal and the positive predictive value of a single elevation of EIA-F, LCMS-F, or LCMS-E is poor. Although EIA-F appears to have better sensitivity in patients with recurrent CD, this study is limited because of the relatively small number of patients. That said, in the 35 patients with Cushing disease, EIA-F and LCMS-E had fewer false negative results than LCMS-F. As previously reported, EIA-F, LCMS-F, or LCMS-E have poor sensitivity for the diagnosis of cortisol excess in patients with adrenal incidentalomas and/or mild cortisol excess [56, 65, 74]. We do not know if adrenal CS patients with elevated late-night salivary cortisol or cortisone have better outcomes after surgical or medical therapy. We have shown that late-night EIA-F does not predict the presence or absence of adrenal insufficiency after unilateral adrenalectomy for adrenal CS [88].

If EIA-F is absurdly elevated, we reflex the sample for measurement of LCMS-F and LCMS-E to assure that contamination with hydrocortisone (authentic cortisol) was not the cause [36]. It seems clear that after dismissing samples with blood contamination by visual inspection, the measurement of transferrin is not of use when EIA-F is only modestly increased.

EIA-F results close to the laboratory upper limit of the reference range may need to be repeated and additional testing such as the low-dose DST performed if the clinical suspicion of CS is significant. A recent multicenter screening study for neoplastic hypercortisolism in a high-risk population employed LNSC (by a platform cortisol immunoassay) and the overnight 1 mg DST for initial diagnostic screening due to the high sensitivity of these tests and their excellent negative predictive value [89]. This study found that 26 (7.4%) of their screened population of 353 patients were found to have neoplastic hypercortisolism. Of these 26 patients, 20 (77%) had ACTH-dependent CS (17 with CD and 3 with ectopic ACTH), and 6 (23%) had ACTH-independent (adrenal) CS. This distribution is very similar to our findings (ACTH-dependent = 40/56 [71%] and ACTH-independent = 16/56 [29%]).

This study has some limitations. We did not address the reasons for the clinical suspicion of CS leading to LNSC testing, so it is not possible to evaluate the pretest probability of neoplastic hypercortisolism. This is also a possible strength of the study, since it mimics real-life clinical practice. Meta-analyses of the diagnostic value of LNSC have shown excellent sensitivity and much better specificity than our data imply [47, 53]. Most studies of LNSC have been done in tertiary endocrine centers with a more selected patient population. Although our findings certainly support the use of LNSC as a screening tool for ACTH-dependent CS, it also highlights the large number of false positive results with a single measurement with EIA-F. Regardless, we can state that LCMS-F and/or LCMS-E do not provide an improvement in sensitivity, although their specificity seem to be better than EIA-F. In addition, this study was dependent on the accuracy of the cutoffs we established contemporaneously in healthy subjects [26]. As with all screening tests, results close to any cutoff should be carefully examined in clinical context with the possibility of false positive or negative results.

We conclude that measurement of late-night salivary cortisol and/or cortisone is a valuable approach to screen patients who are ultimately found to have ACTH-dependent hypercortisolism and that EIA-F and LCMS-E have excellent sensitivity and negative
predictive value in that regard. Nonetheless, false positive results are common in patients
with suspected hypercortisolism (particularly those with fewer and less severe features)
and, therefore, these measurements give suboptimal specificity and positive predictive
values in a real-life patient population [54]. Clearly, biochemical screening for neoplastic
hypercortisolism should take into consideration the pretest probability of this disorder. For
example, a prior history of pituitary surgery for CD certainly increases the posttest proba-
bility of recurrent CD in a patient with an elevated salivary cortisol and/or cortisone [86].
On the other hand, the measurement is not a valuable screening tool in patients with an
incidental adrenal nodule and possible mild cortisol excess. In Table 7, we have summarized
our approach to screen patients for CS, taking into account the findings of the current study
and our clinical experience with this enigmatic disease. The clinical index of suspicion of
an experienced endocrinologist complemented by a systematic and logical biochemical diag-
nostic approach, are necessary to secure a diagnosis of this protean and complex disorder.

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Novartis Pharmaceuticals. B.J. has been a research investigator for Novartis Pharmaceuticals.
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Table 7. A Sensible Approach to Screening for Neoplastic Hypercortisolism

| Approach                                                                 |
|--------------------------------------------------------------------------|
| • Comprehensive history and careful examination                            |
| • Consider clinical and biochemical presentation and pretest probability   |
| • Late-night salivary cortisol (LNSC)—at least two                       |
|   • Excludes ACTH-dependent cortisol excess >95% of the time              |
|   • Predicts recurrence of surgically treated Cushing disease             |
|     • With basal plasma ACTH levels within or above reference interval   |
|     • As a routine annual screen in patients in remission                |
| • Adrenal nodular disease (with possible cortisol excess)                 |
|   • Overnight dexamethasone suppression test (DST) preferred              |
| • discordant or confusing results                                        |
|   • Repeat testing (LNSC, DST, UFC).                                     |
|   • Consider preanalytical error (eg, sample contamination or incorrect collection) |

UFC, 24-hour urinary free cortisol
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