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

Joshua Kannankeril,[1] Ty Carroll,[2] James W. Findling,[2] Bradley Javorsky,[2] Ian L. Gunsolus,[3] Jonathan Phillips,[4] and Hershel Raff[1,4]

[1]*Division of Endocrinology and Molecular Medicine, Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin, 53226;* [2]*Endocrinology Center and Clinics, Medical College of Wisconsin, Milwaukee, Wisconsin 53051;* [3]*Department of Pathology, Medical College of Wisconsin, Milwaukee, Wisconsin, 53226; and* [4]*Endocrine Research Laboratory, Aurora St. Luke's Medical Center, Advocate Aurora Research Institute, Milwaukee, Wisconsin 53215*

ORCiD numbers: 0000-0003-4797-4219 (J. Kannankeril); 0000-0001-5052-3043 (B. Javorsky); 0000-0002-5128-8476 (H. Raff).

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 specificity 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.

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
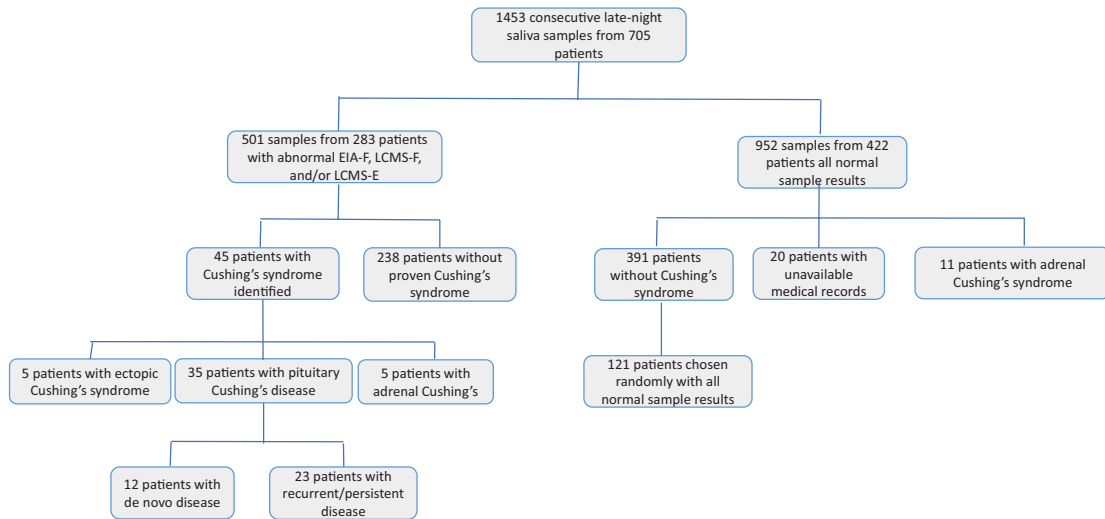
Figure 1. Flow chart showing late-night salivary samples and associated patients in the study.

(EIA-F, LCMS-F, and/or LCMS-E). 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 cortisone (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] ug/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

Table 1. Patient Data

	No increased salivary results		At least one increased salivary result		
	Normal Patients	**Adrenal Cushing Syndrome**	**Cushing Disease**	**Ectopic ACTH**	**Adrenal Cushing Syndrome**
	(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/m^2	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	4/1	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.
[a]Different from all other groups by Mann-Whitney rank sum test (*P* = 0.008-0.032). [b]Different from normal by Mann-Whitney rank sum test (*P* = 0.006). [c]Different 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 + Above - Below	ACTH-dependent Cushing Syndrome		Sensitivity	Specificity	Positive Predictive Value (PPV)	Negative Predictive Value (NPV)
	Yes	**No**				
EIA-F +/-	39/1	193/436	97.5 (86.8-99.9)	69.3 (65.6-72.9)	16.8 (15.1-18.7)	99.8 (98.4-99.9)
LCMS-F +/-	27/13	97/532	67.5 (50.9-82.4)	84.6 (81.5-87.3)	21.8 (17.4-27.0)	97.6 (96.3-98.5)
LCMS-E +/-	37/3	150/479	92.5 (72.6-98.4)	76.2 (72.6-79.4)	19.8 (17.3-22.5)	99.4 (97.9-99.7)

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.

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

Sensitivity, specificity, PPV, and NPV are in percentages

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

Abnormal Result	Proven CS				CS not proven (238)
	Total (45)	CD (35)	Ectopic (5)	Adrenal (5)	
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.
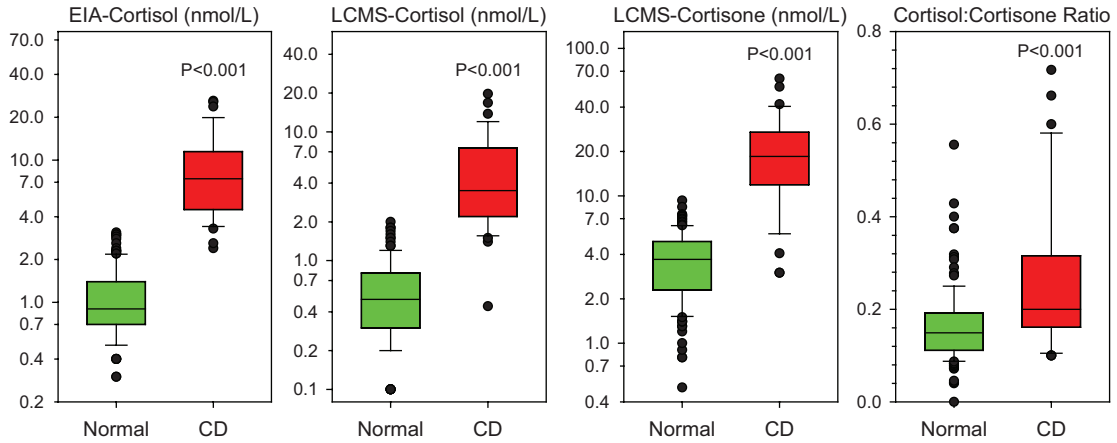
Figure 2. Late-night salivary enzyme immunoassay cortisol (EIA-Cortisol), liquid chromatography-tandem mass spectrometry (LCMS) cortisol and cortisone, and the ratio of LCMS cortisol to cortisone in 35 patients with Cushing disease compared with the 121 patients randomly chosen with all normal salivary 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.
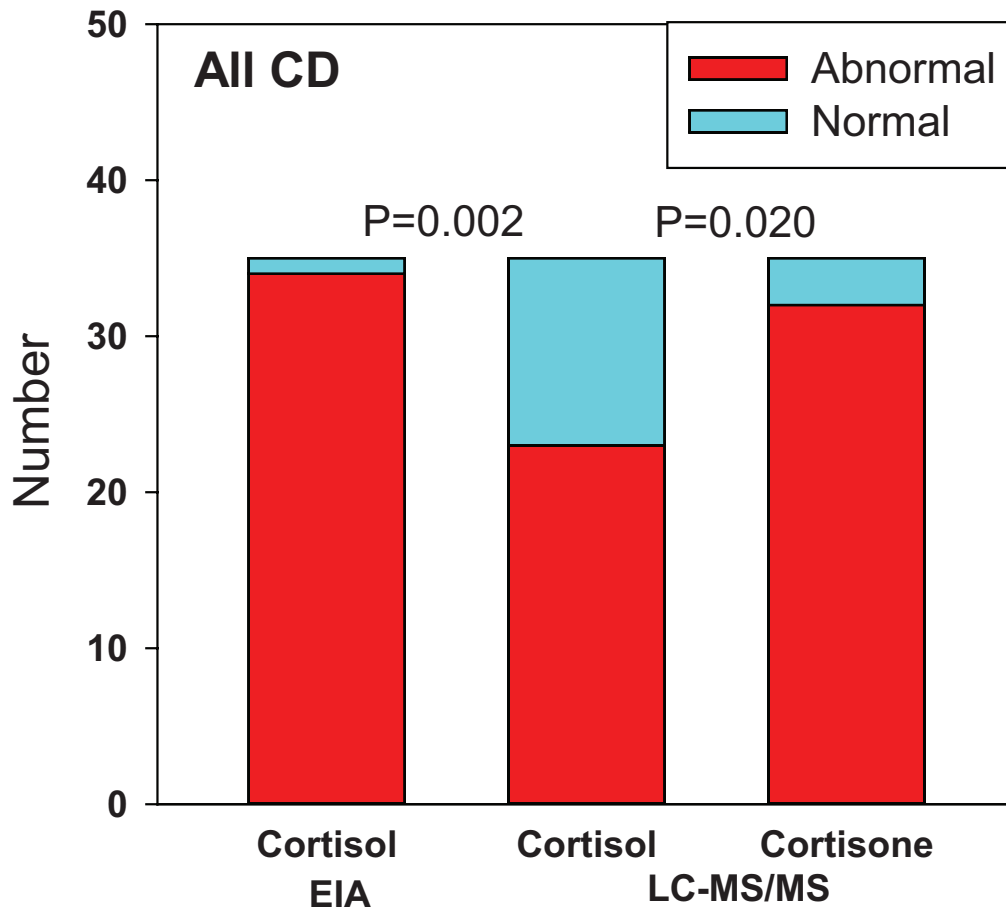

Figure 3. Number of Cushing disease patients (N = 35) with late-night EIA-Cortisol and LC-MS/MS cortisol and cortisone equal to or below (Normal) or above (Abnormal) the reference range for that assay [26]. *P* values are from chi-square analysis.

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) (N = 12)	Median (IQR) (N = 23)	P values
Age, years	47 (33-61)	51 (38-68)	0.375
BMI, kg/m^2	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.
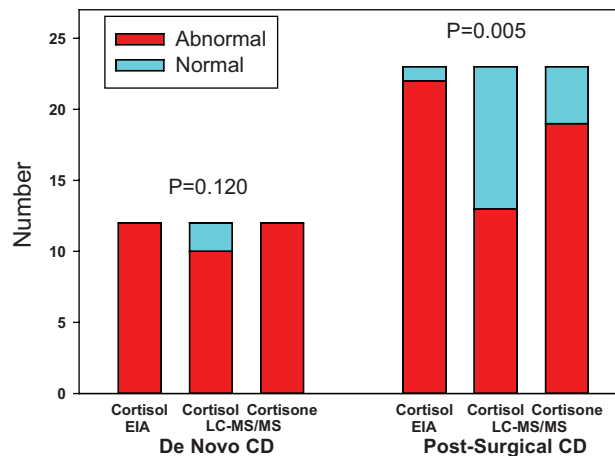

Figure 4. Number of Cushing disease patients with *de novo* Cushing disease (N = 12) and patients with recurrent/persistent Cushing disease (N = 23) with late-night EIA-Cortisol and LC-MS/MS cortisol and cortisone equal to or below (Normal) or above (Abnormal) the reference range for that assay [26].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

Table 6. 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

	Salivary cortisol to cortisone ratio		
	<1	≥1	
	(n = 19)	(n = 20)	P values
Transferrin	0.50 [0.30-1.2]	0.75 [0.25-1.40]	0.725
EIA-Cortisol	10.2 [5.2]	10.1 [6.8]	0.976
LCMS-Cortisol	5.2 [2.8]	7.1 [5.2]	0.172
LCMS-Cortisone	20.0 [11.5]	3.1 [2.4]	<0.001
Cortisol/Cortisone ratio	0.24 [0.19-0.34]	2.23 [1.64-3.18]	<0.002

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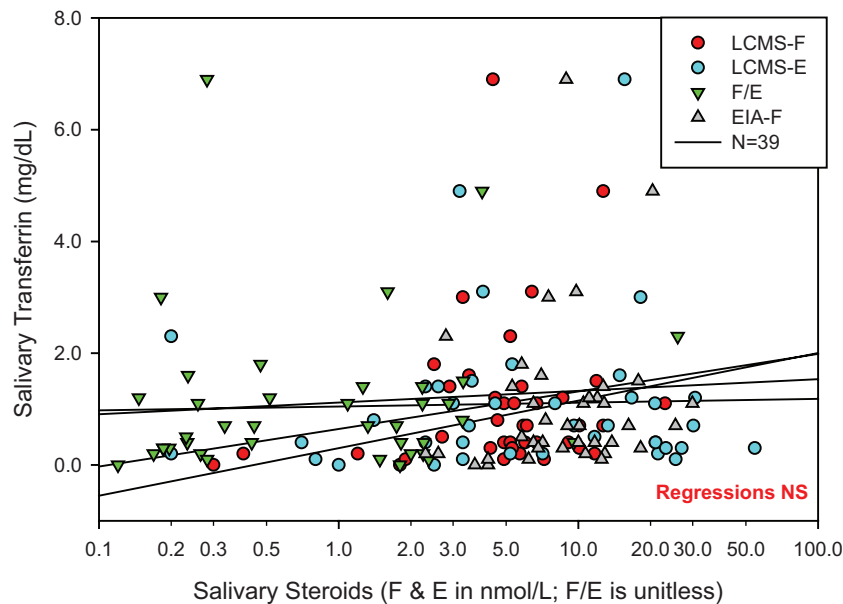

Figure 5. Correlation of salivary steroid measurements vs salivary transferrin concentrations in 39 patients from Table 6. There were no significant correlations found.

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

[78, 80, 82, 83]. 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 LCMC-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

---

Table 7.   A Sensible Approach to Screening for Neoplastic Hypercortisolism

---

- 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

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 probability 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 diagnostic approach, are necessary to secure a diagnosis of this protean and complex disorder.

Acknowledgments

Additional Information

Correspondence: Hershel Raff PhD, Endocrine Research Laboratory, Aurora St. Luke's Medical Center, 2801 W Kk River Pky Suite 260, Milwaukee, WI 53215, USA. E-mail: hraff@mcw.edu.
Disclosure Summary: J.W.F. has received consulting fees from Corcept Therapeutics, and Novartis Pharmaceuticals and has been a research investigator for Corcept Therapeutics and Novartis Pharmaceuticals. H.R. has received consulting fees and research reagents from Corcept Therapeutics, and consulting fees and a research grant from Cerium Pharmaceuticals. T.C. has received consulting fees from Corcept Therapeutics and has been a research investigator for Novartis Pharmaceuticals. B.J. has been a research investigator for Novartis Pharmaceuticals. I.L.G. has been a research investigator for Abbott Laboratories. The remaining authors have nothing to disclose.

Data Availability: All data generated or analyzed during this study are included in this published article.

References

1. Arnaldi G, Angeli A, Atkinson AB, et al. Diagnosis and complications of Cushing's syndrome: a consensus statement. *J Clin Endocrinol Metab.* 2003;**88**:5593-5602

2. Lacroix A, Feelders RA, Stratakis CA, Nieman LK. Cushing's syndrome. *Lancet.* 2015;**386**(9996):913-927.

3. Lonser RR, Nieman L, Oldfield EH. Cushing's disease: pathobiology, diagnosis, and management. *J Neurosurg.* 2017;**126**(2):404-417.

4. Newell-Price J, Bertagna X, Grossman AB, Nieman LK. Cushing's syndrome. *Lancet.* 2006;**367**(9522):1605-1617.

5. Nieman LK. Cushing's syndrome: update on signs, symptoms and biochemical screening. *Eur J Endocrinol.* 2015;**173**(4):M33-M38.

6. Isidori AM, Graziadio C, Paragliola RM, et al. The hypertension of Cushing's syndrome: controversies in the pathophysiology and focus on cardiovascular complications. *J Hypertens.* 2015;**33**(1):44-60.

7. Isidori AM, Lenzi A. Ectopic ACTH syndrome. *Arq Bras Endocrinol Metabol.* 2007;**51**(8):1217-1225.

8. Barbot M, Zilio M, Scaroni C. Cushing's syndrome: overview of clinical presentation, diagnostic tools and complications. *Best Pract Res Clin Endocrinol Metab*. 2020;**34**(2):101380.

9. Bourdeau I, Bard C, Noël B, et al. Loss of brain volume in endogenous Cushing's syndrome and its reversibility after correction of hypercortisolism. *J Clin Endocrinol Metab.* 2002;**87**(5):1949-1954.

10. Pivonello R, Simeoli C, De Martino MC, et al. Neuropsychiatric disorders in Cushing's syndrome. *Front Neurosci.* 2015;**9**:129

11. Starkman MN. Neuropsychiatric findings in Cushing syndrome and exogenous glucocorticoid administration. *Endocrinol Metab Clin North Am.* 2013;**42**(3):477-488.

12. Starkman MN, Gebarski SS, Berent S, Schteingart DE. Hippocampal formation volume, memory dysfunction, and cortisol levels in patients with Cushing's syndrome. *Biol Psychiatry.* 1992;**32**(9):756-765.

13. Starkman MN, Schteingart DE. Neuropsychiatric manifestations of patients with Cushing's syndrome. Relationship to cortisol and adrenocorticotropic hormone levels. *Arch Intern Med.* 1981;**141**(2):215-219.

14. dos Santos CV, Vieira Neto L, Madeira M, et al. Bone density and microarchitecture in endogenous hypercortisolism. *Clin Endocrinol (Oxf).* 2015;**83**(4):468-474.

15. Raff H, Carroll T. Cushing's syndrome: from physiological principles to diagnosis and clinical care. *J Physiol.* 2015;**593**(3):493-506.

16. Raff H, Sharma ST, Nieman LK. Physiological basis for the etiology, diagnosis, and treatment of adrenal disorders: Cushing's syndrome, adrenal insufficiency, and congenital adrenal hyperplasia. *Compr Physiol.* 2014;**4**(2):739-769.

17. Dekkers OM, Horváth-Puhó E, Jørgensen JO, et al. Multisystem morbidity and mortality in Cushing's syndrome: a cohort study. *J Clin Endocrinol Metab.* 2013;**98**(6):2277-2284.

18. Nieman LK. Recent updates on the diagnosis and management of cushing's syndrome. *Endocrinol Metab (Seoul).* 2018;**33**(2):139-146.

19. Chiodini I, Albani A, Ambrogio AG, et al. Six controversial issues on subclinical Cushing's syndrome. *Endocrine.* 2017;**56**(2):262-266.

20. Delivanis DA, Athimulam S, Bancos I. Modern management of mild autonomous cortisol secretion. *Clin Pharmacol Ther.* 2019;**106**(6):1209-1221.

21. Feelders RA, Hofland LJ. Medical treatment of Cushing's disease. *J Clin Endocrinol Metab.* 2013;**98**(2):425-438.

22. Clayton RN, Raskauskiene D, Reulen RC, Jones PW. Mortality and morbidity in Cushing's disease over 50 years in Stoke-on-Trent, UK: audit and meta-analysis of literature. *J Clin Endocrinol Metab.* 2011;**96**(3):632-642.

23. Nieman LK, Biller BM, Findling JW, et al. Treatment of Cushing's syndrome: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab.* 2015;**100**(8):2807-2831.

24. Nieman LK, Biller BM, Findling JW, et al. The diagnosis of Cushing's syndrome: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab.* 2008;**93**(5):1526-1540.

25. Machado MC, Fragoso MC, Moreira AC, et al. Recommendations of the neuroendocrinology department of the Brazilian society of endocrinology and metabolism for the diagnosis of Cushing's disease in Brazil. *Arch Endocrinol Metab.* 2016;**60**(3):267-286.

26. Raff H, Phillips JM. Bedtime salivary cortisol and cortisone by LC-MS/MS in healthy adult subjects: evaluation of sampling time. *J Endocr Soc.* 2019;**3**(8):1631-1640.

27. Raff H, Homar PJ, Skoner DP. New enzyme immunoassay for salivary cortisol. *Clin Chem.* 2003;**49**(1):203-204.

28. Miller R, Plessow F, Rauh M, Gröschl M, Kirschbaum C. Comparison of salivary cortisol as measured by different immunoassays and tandem mass spectrometry. *Psychoneuroendocrinology.* 2013;**38**(1):50-57.

29. Casals G, Hanzu FA. Cortisol measurements in Cushing's syndrome: immunoassay or mass spectrometry? *Ann Lab Med*. 2020;**40**(4):285-296.

30. Perogamvros I, Keevil BG, Ray DW, Trainer PJ. Salivary cortisone is a potential biomarker for serum free cortisol. *J Clin Endocrinol Metab*. 2010;**95**(11):4951-4958.

31. Perogamvros I, Owen LJ, Newell-Price J, Ray DW, Trainer PJ, Keevil BG. Simultaneous measurement of cortisol and cortisone in human saliva using liquid chromatography-tandem mass spectrometry: application in basal and stimulated conditions. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009;**877**(29):3771-3775.

32. Erickson D, Singh RJ, Sathananthan A, Vella A, Bryant SC. Late-night salivary cortisol for diagnosis of Cushing's syndrome by liquid chromatography/tandem mass spectrometry assay. *Clin Endocrinol (Oxf)*. 2012;**76**(4):467-472.

33. Zerikly RK, Amiri L, Faiman C, et al. Diagnostic characteristics of late-night salivary cortisol using liquid chromatography-tandem mass spectrometry. *J Clin Endocrinol Metab*. 2010;**95**(10):4555-4559.

34. Raff H. Update on late-night salivary cortisol for the diagnosis of Cushing's syndrome: methodological considerations. *Endocrine*. 2013;**44**(2):346-349.

35. Raff H. Cushing syndrome: update on testing. *Endocrinol Metab Clin North Am*. 2015;**44**(1):43-50.

36. Raff H, Singh RJ. Measurement of late-night salivary cortisol and cortisone by LC-MS/MS to assess preanalytical sample contamination with topical hydrocortisone. *Clin Chem*. 2012;**58**(5):947-948.

37. RRID:AB_2801306. https://scicrunch.org/resolver/AB_2801306.

38. Schwartz EB, Granger DA. Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clin Chem*. 2004;**50**(3):654-656.

39. Kivlighan KT, Granger DA, Schwartz EB, Nelson V, Curran M, Shirtcliff EA. Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Horm Behav*. 2004;**46**(1):39-46.

40. MEDCALC. https://www.medcalc.org/calc/diagnostic_test.php.

41. Raff H. Measurement of salivary cortisone to assess the adequacy of hydrocortisone replacement. *J Clin Endocrinol Metab*. 2016;**101**(4):1350-1352.

42. Findling JW, Raff H. Cushing's Syndrome: important issues in diagnosis and management. *J Clin Endocrinol Metab*. 2006;**91**(10):3746-3753.

43. Findling JW, Raff H. Diagnosis and differential diagnosis of Cushing's syndrome. *Endocrinol Metab Clin North Am*. 2001;**30**(3):729-747.

44. Findling JW, Raff H. Newer diagnostic techniques and problems in Cushing's disease. *Endocrinol Metab Clin North Am*. 1999;**28**(1):191-210.

45. Findling JW, Raff H. Screening and diagnosis of Cushing's syndrome. *Endocrinol Metab Clin North Am*. 2005;**34**(2):385-402, ix.

46. Carroll T, Raff H, Findling JW. Late-night salivary cortisol measurement in the diagnosis of Cushing's syndrome. *Nat Clin Pract Endocrinol Metab*. 2008;**4**(6):344-350.

47. Carroll T, Raff H, Findling JW. Late-night salivary cortisol for the diagnosis of Cushing syndrome: a meta-analysis. *Endocr Pract*. 2009;**15**(4):335-342.

48. Raff H. Cushing's syndrome: diagnosis and surveillance using salivary cortisol. *Pituitary*. 2012;**15**(1):64-70.

49. Raff H. The role of salivary cortisol determinations in the diagnosis of Cushing's syndrome. *Curr Opin Endocrinol Diabetes*. 2004;**11**:271-275

50. Raff H. Salivary cortisol: a useful measurement in the diagnosis of Cushing's syndrome and the evaluation of the hypothalamic-pituitary adrenal axis. *Endocrinol*. 2000;**10**:9-17

51. Raff H, Findling JW. A physiologic approach to diagnosis of the Cushing syndrome. *Ann Intern Med*. 2003;**138**(12):980-991.

52. Raff H, Raff JL, Findling JW. Late-night salivary cortisol as a screening test for Cushing's syndrome. *J Clin Endocrinol Metab*. 1998;**83**(8):2681-2686.

53. Galm BP, Qiao N, Klibanski A, Biller BMK, Tritos NA. Accuracy of laboratory tests for the diagnosis of Cushing syndrome. *J Clin Endocrinol Metab*. 2020;**105**(6): 2081–2094.

54. Ceccato F, Marcelli G, Martino M, et al. The diagnostic accuracy of increased late night salivary cortisol for Cushing's syndrome: a real-life prospective study. *J Endocrinol Invest*. 2019;**42**(3):327-335.

55. Antonelli G, Ceccato F, Artusi C, Marinova M, Plebani M. Salivary cortisol and cortisone by LC-MS/MS: validation, reference intervals and diagnostic accuracy in Cushing's syndrome. *Clin Chim Acta*. 2015;**451**(Pt B):247-251.

56. Ceccato F, Barbot M, Albiger N, et al. Daily salivary cortisol and cortisone rhythm in patients with adrenal incidentaloma. *Endocrine*. 2018;**59**:510-519

57. Bäcklund N, Brattsand G, Israelsson M, et al. Reference intervals of salivary cortisol and cortisone and their diagnostic accuracy in Cushing's syndrome. *Eur J Endocrinol.* 2020;**182**(6):569-582.

58. Mészáros K, Karvaly G, Márta Z, et al. Diagnostic performance of a newly developed salivary cortisol and cortisone measurement using an LC-MS/MS method with simple and rapid sample preparation. *J Endocrinol Invest.* 2018;**41**(3):315-323.

59. Debono M, Harrison RF, Whitaker MJ, et al. Salivary cortisone reflects cortisol exposure under physiological conditions and after hydrocortisone. *J Clin Endocrinol Metab.* 2016;**101**(4):1469-1477.

60. Harrison RF, Debono M, Whitaker MJ, Keevil BG, Newell-Price J, Ross RJ. Salivary cortisone to estimate cortisol exposure and sampling frequency required based on serum cortisol measurements. *J Clin Endocrinol Metab.* 2019;**104**(3):765-772.

61. Findling JW, Raff H. Diagnosis of endocrine disease: differentiation of pathologic/neoplastic hypercortisolism (Cushing's syndrome) from physiologic/non-neoplastic hypercortisolism (formerly known as pseudo-Cushing's syndrome). *Eur J Endocrinol.* 2017;**176**(5):R205-R216.

62. Ceccato F, Antonelli G, Frigo AC, et al. First-line screening tests for Cushing's syndrome in patients with adrenal incidentaloma: the role of urinary free cortisol measured by LC-MS/MS. *J Endocrinol Invest.* 2017;**40**(7):753-760.

63. Ceccato F, Barbot M, Zilio M, et al. Performance of salivary cortisol in the diagnosis of Cushing's syndrome, adrenal incidentaloma, and adrenal insufficiency. *Eur J Endocrinol.* 2013;**169**(1): 31-36.

64. Fassnacht M, Arlt W, Bancos I, et al. Management of adrenal incidentalomas: European society of endocrinology clinical practice guideline in collaboration with the European network for the study of adrenal tumors. *Eur J Endocrinol.* 2016;**175**(2):G1-G34.

65. Masserini B, Morelli V, Bergamaschi S, et al. The limited role of midnight salivary cortisol levels in the diagnosis of subclinical hypercortisolism in patients with adrenal incidentaloma. *Eur J Endocrinol.* 2009;**160**(1):87-92.

66. Nieman LK. Approach to the patient with an adrenal incidentaloma. *J Clin Endocrinol Metab.* 2010;**95**(9):4106-4113.

67. Nunes ML, Vattaut S, Corcuff JB, et al. Late-night salivary cortisol for diagnosis of overt and subclinical Cushing's syndrome in hospitalized and ambulatory patients. *J Clin Endocrinol Metab.* 2009;**94**(2):456-462.

68. Palmieri S, Morelli V, Polledri E, et al. The role of salivary cortisol measured by liquid chromatography-tandem mass spectrometry in the diagnosis of subclinical hypercortisolism. *Eur J Endocrinol.* 2013;**168**(3):289-296.

69. Reincke M. Subclinical Cushing's syndrome. *Endocrinol Metab Clin North Am.* 2000;**29**(1):43-56.

70. Sasaki Y, Katabami T, Asai S, Fukuda H, Tanaka Y. In the overnight dexamethasone suppression test, 1.0 mg loading is superior to 0.5 mg loading for diagnosing subclinical adrenal Cushing's syndrome based on plasma dexamethasone levels determined using liquid chromatography-tandem mass spectrometry. *Endocr J.* 2017;**64**(9):833-842.

71. Starker LF, Kunstman JW, Carling T. Subclinical Cushing syndrome: a review. *Surg Clin North Am.* 2014;**94**(3):657-668.

72. Terzolo M, Bovio S, Reimondo G, et al. Subclinical Cushing's syndrome in adrenal incidentalomas. *Endocrinol Metab Clin North Am.* 2005;**34**(2):423-39, x.

73. Terzolo M, Stigliano A, Chiodini I, et al. AME position statement on adrenal incidentaloma. *Eur J Endocrinol.* 2011;**164**(6):851-870.

74. Chiodini I, Ramos-Rivera A, Marcus AO, Yau H. Adrenal Hypercortisolism: a closer look at screening, diagnosis, and important considerations of different testing modalities. *J Endocr Soc.* 2019;**3**(5):1097-1109.

75. Elhassan YS, Alahdab F, Prete A, et al. Natural history of adrenal incidentalomas with and without mild autonomous cortisol excess: a systematic review and meta-analysis. *Ann Intern Med.* 2019;**171**(2):107-116.

76. Masjkur J, Gruber M, Peitzsch M, et al. Plasma steroid profiles in subclinical compared with overt adrenal cushing syndrome. *J Clin Endocrinol Metab.* 2019;**104**:4331-4340

77. Raff H, Cohen EP, Findling JW. A commentary on diagnosing Cushing's disease in the context of renal failure. *Eur J Endocrinol.* 2019;**181**(4):C9-C11.

78. Amlashi FG, Swearingen B, Faje AT, et al. Accuracy of late-night salivary cortisol in evaluating postoperative remission and recurrence in Cushing's disease. *J Clin Endocrinol Metab.* 2015;**100**(10):3770-3777.

79. Sheth SA, Bourne SK, Tritos NA, Swearingen B. Neurosurgical treatment of Cushing disease. *Neurosurg Clin N Am.* 2012;**23**(4):639-651.

80. Carrasco CA, Coste J, Guignat L, et al. Midnight salivary cortisol determination for assessing the outcome of transsphenoidal surgery in Cushing's disease. *J Clin Endocrinol Metab.* 2008;**93**(12):4728-4734.

81. Hinojosa-Amaya JM, Varlamov EV, McCartney S, Fleseriu M. Hypercortisolemia Recurrence in Cushing's disease; a diagnostic challenge. *Front Endocrinol (Lausanne).* 2019;**10**:740.

82. Fleseriu M, Hamrahian AH, Hoffman AR, Kelly DF, Katznelson L; AACE Neuroendocrine and Pituitary Scientific Committee. American association of clinical endocrinologists and american college of endocrinology disease state clinical review: diagnosis of recurrence in Cushing disease. *Endocr Pract.* 2016;**22**(12):1436-1448.

83. Danet-Lamasou M, Asselineau J, Perez P, et al. Accuracy of repeated measurements of late-night salivary cortisol to screen for early-stage recurrence of Cushing's disease following pituitary surgery. *Clin Endocrinol (Oxf).* 2015;**82**(2):260-266.

84. Geer EB, Ayala A, Bonert V, et al. Follow-up intervals in patients with Cushing's disease: recommendations from a panel of experienced pituitary clinicians. *Pituitary.* 2017;**20**:422-429

85. Geer EB, Shafiq I, Gordon MB, et al. Biochemical control during long-term follow-up of 230 adult patients with Cushing disease: a multicenter retrospective study. *Endocr Pract.* 2017;**23**:962-970

86. Carroll TB, Javorsky BR, Findling JW. Postsurgical recurrent Cushing disease: clinical benefit of early intervention in patients with normal urinary free cortisol. *Endocr Pract.* 2016;**22**(10):1216-1223.

87. Nieman LK. Diagnosis of Cushing's syndrome in the modern era. *Endocrinol Metab Clin North Am.* 2018;**47**(2):259-273.

88. Ortiz DI, Findling JW, Carroll TB, et al. Cosyntropin stimulation testing on postoperative day 1 allows for selective glucocorticoid replacement therapy after adrenalectomy for hypercortisolism: results of a novel, multidisciplinary institutional protocol. *Surgery.* 2016;**159**(1):259-265.

89. Leon-Justel A, Madrazo-Atutxa A, Alvarez-Rios AI, et al. A probabilistic model for Cushing's syndrome screening in at-risk populations: a prospective multicenter study. *J Clin Endocrinol Metab.* 2016;**101**:3747-3754.