Endocrine (2012) 41:494–500
DOI 10.1007/s12020-012-9658-3

ORIGINAL ARTICLE

Diagnostic performance of late-night salivary cortisol measured by automated electrochemiluminescence immunoassay in obese and overweight patients referred to exclude Cushing's syndrome

Zhanna E. Belaya · Alexander V. Iljin · Galina A. Melnichenko · Liudmila Y. Rozhinskaya · Natalia V. Dragunova · Larisa K. Dzeranova · Svetlana A. Butrova · Ekaterina A. Troshina · Ivan I. Dedov

Received: 5 January 2012 / Accepted: 8 March 2012 / Published online: 25 March 2012

Abstract This study estimates diagnostic performance of late-night salivary cortisol (LNSC) as measured by automated electrochemiluminescence immunoassay (ECLIA), evaluates the clinical implication of two consecutive LNSC measurements, and compares its accuracy with enzyme-linked immunosorbent assay (ELISA) and serum cortisol after low-dose dexamethasone suppression test (DST) in obese and overweight patients referred for suspected Cushing's syndrome (CS). One hundred twenty three consecutive obese and overweight referred patients and 98 healthy volunteers provided two saliva samples collected at 23:00 using a Salivette (Sarstedt, Germany), assayed by ECLIA (Cobas e601) and ELISA. The patients underwent DST and were further evaluated until CS was pathologically confirmed ($n = 45$) or excluded. Diagnostic performance of LNSC was evaluated by receiver operating characteristic (ROC) analysis. The total areas under the curve (AUC) were calculated to compare the different tests. We found that a cut-off value of 9.4 nmol/l can differentiate CS among obese and overweight patients with sensitivity of 84.4 % (95% CI 71.2–92.2), specificity of 92.3 % (95% CI 84.2–96.4), and diagnostic odds ratio of 65.1 (95% CI 20.4–207.6). No difference was found between AUCs from the first, second, and the mean from the two LNSC measurements (ECLIA), LNSC (ELISA), or DST. The single LNSC (ECLIA) and DST improved the sensitivity and specificity for concordant results up to 100 and 97.4 %, respectively. In conclusion, due to its automation and its comparable diagnostic performance, ECLIA is preferable as a first-line LNSC screening test for CS. The initial use of single LNSC followed by DST provides better diagnostic performance for concordant results.

Keywords Cushing's syndrome · Late-night salivary cortisol · Electrochemiluminescence immunoassay · Enzyme-linked immunosorbent assay · Obesity

Z. E. Belaya (✉) · A. V. Iljin · G. A. Melnichenko · L. Y. Rozhinskaya · N. V. Dragunova · L. K. Dzeranova · S. A. Butrova · E. A. Troshina · I. I. Dedov
The National Research Center for Endocrinology, Dmitria Uljanova, 11, Moscow, Russia 117036
e-mail: jannabelaya@gmail.com

Current obesity and metabolic syndrome epidemics have increased the number of patients with the Cushing's syndrome (CS) phenotype. Although CS is rare [1], 2–5 % of patients with poorly-controlled diseases such as hypertension, diabetes mellitus, and idiopathic osteoporosis have previously undiagnosed CS [2–6]. CS should be excluded among patients with adrenal incidentaloma [7, 8].

For patients suspected of having CS, current clinical guidelines recommend the initial use of one of the following high-accuracy tests: 24-h urinary free cortisol (24-h UFC), late-night salivary cortisol (LNSC), and serum cortisol after 1 mg overnight dexamethasone suppression test (DST) [9]. LNSC seems the best choice for the first test, compared to DST and 24-h UFC, because it is non-invasive, less time-consuming, and has a stress-free, easy-collection method performed by the patient. Saliva contains stable cortisol [10, 11], unaffected by alterations in cortisol-binding globulin (e.g., during treatment with oral contraceptives) [12]. The feasibility and diagnostic performance of using LNSC [radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA)] to clinically evaluate CS has been studied since the 1980s [13–20]. However, introducing LNSC measurement into clinical practice is challenging because there is no

consensus regarding LNSC cut-off values (ranging from 2.8 nmol/l to 15.2 nmol/l) [21]. Different assays for measuring LNSC have used different reference ranges and cut-off values [22, 23]. Notably, some conditions such as obesity might influence diagnostic performance of assays. A screening of 369 obese and overweight patients who indicated on a questionnaire the presence of at least two signs or symptoms of CS has shown that predefined reference ranges (LNSC measured by RIA and liquid chromatography-tandem mass spectrometry) for such may be falsely abnormal, revealing how challenging it is to establish CS among obese patients [24]. After thorough investigation, no CS was confirmed in this study [24] and it could thusly be concluded that widespread screening of obese and overweight patients who report by themselves symptoms of CS should not be recommended [24]. However, weight gain and obesity are the most frequent symptoms of CS and should not be overlooked [25].

Taking advantage of our position as the primary referral clinic for patients with suspected CS, we enrolled a sizeable population with a high percentage of CS to estimate diagnostic performance of LNSC as measured by automated electrochemiluminescence immunoassay (ECLIA), to evaluate the clinical implication of two consecutive LNSC measurements, and to compare its accuracy with ELISA and DST in obese and overweight patients referred by physicians to exclude CS.

Subjects and methods

The Institutional Review Board of the National Research Center for Endocrinology (NRCE) approved the study protocol.

Patients with clinical findings suggestive of CS were unrestrictedly referred to our clinic by clinicians from Moscow and other regions of Russia between January 2010 and January 2011.

Consecutive patients who were suspected of having CS and who complained of obesity, body mass index (BMI) $\geq$30 kg/m^2, or very fast weight gain to a BMI of 26–29 kg/m^2 were invited to participate.

Patients who gave informed consent were enrolled in the study ($n = 127$). Only the data of patients whose diagnosis was confirmed during the study were eventually analyzed and presented ($n = 123$).

To explore normative data for the assay, we recruited 98 healthy volunteers from the interns/staff/faculty population of NRCE. Healthy volunteers did not have any complaints regarding their health, had regular preventive examinations that certified them as generally healthy and did not have signs or symptoms suspicious of CS after examination by an experienced endocrinologist.

Exclusion criteria

Pregnancy, shift workers, glucocorticoid use, alcohol abuse, gingival bleeding, acute infection, exacerbation of chronic disease, severe conditions (i.e., renal and liver insufficiency, heart attack, stroke, and terminal conditions), and mental insanity.

All healthy volunteers were outpatients. The patients from the referred population were mainly outpatients and partly inpatients. Two studies directly comparing outpatient and inpatient settings for LNSC [15, 26] showed no need for using specific cut-off values for ambulatory and hospitalized patients.

Diagnostic evaluations followed recent clinical practice guidelines [9]. The initial use of LNSC measured by ECLIA and then ELISA (cut-off value: 4 nmol/l) was independently followed by DST (cut-off value for suppression: 50 nmol/l) [9]. The majority of patients underwent a third or even fourth test: 24 h UFC (reference range 60–413 nmol/24 h) ($n = 83$) and/or awake serum cortisol at 23:00 (reference range 46–270 nmol/l) ($n = 57$).

CS diagnosis was established in 45 cases. Adrenal CS was confirmed and surgically treated in four cases. A bronchial carcinoid tumor was successfully removed in one case. Cushing's disease was diagnosed in 40 cases, and the patients underwent transsphenoidal adenomectomy. After surgery, the material was evaluated by the histologist in all 45 cases. Remission was not achieved in eight cases of Cushing's disease after the first surgery. Four patients benefited from a second surgery, one patient died from sepsis and three others underwent radiation therapy. The histological material obtained was sufficient to confirm CS in these patients.

CS was excluded in 78 patients after two initial tests (LNSC; DST) and at least one additional confirmatory test: normal 24 h UFC and/or normal awake serum cortisol collected at 23:00. Patients were under observation for an average of 6 months (min: 3 months; max: 12 months) in our center, or in the practice of the referring physicians, and received appropriate treatment for constitutional obesity, during which we could observe the absence of progression toward overt CS.

In four cases, we could not positively verify the diagnosis, and the data was excluded.

Subjects provided saliva samples at 23:00 on two separate days using a commercially-available salivary sampling device (Salivette; Sarstedt). Written instructions were given to every participant. All participants were advised to avoid physically or emotionally stressful conditions before taking their samples, to avoid alcohol consumption for at least 24 h, and to not eat, drink, brush teeth, or smoke for at least 30 min before the saliva collection. After chewing a cotton swab for approximately 2–3 min, the swab was

returned in a small tube and placed at 4–8 °C by the subjects. The specimens were brought to the laboratory within 2 days of collection. The tubes were centrifuged at 2,000 rpm for 5 min. After removing the cotton swab, the collected saliva samples were assayed by ECLIA Cobas e 601 (Cortisol no. 11875116 122, Roche). The remaining saliva from 197 subjects was stored at −70 °C and assayed by ELISA (DRG Salivary Cortisol ELISA KIT SLV-2930).

Patients from the referred population underwent DST. At midnight, after the second saliva sample was collected, patients received 1 mg of dexamethasone. Cortisol serum samples were taken the following morning and assayed by ECLIA Cobas e 601 (Cortisol Nº 11875116 122, Roche), which was also used to assay awake serum cortisol at 23:00.

UFC at 24 h was measured by an immunochemiluminescence assay (extraction with diethyl ether) on a Vitros ECi.

Statistical analysis

Descriptive statistics Quantitative parameters were presented as means, standard deviations, medians, and ranges, qualitative parameters were presented as percentages and binomial 95% confidence intervals.

Since normality tests (skewness and curtosis) rejected normality for the majority of quantitative parameters, non-parametric test were utilized. The Mann–Whitney test was utilized to compare quantitative parameters in two independent samples; paired Wilcoxon test was used to compare quantitative parameters in two related samples. A two-tailed approach for calculation of p was utilized. A p value <0.05 was considered statistically significant. Spearman's rank test was used for correlations. Reference ranges were calculated for the data from healthy participants and presented as the 2.5–97.5th percentiles. The cut-off value (threshold) for LNSC was chosen to achieve maximum diagnostic accuracy [maximum sum of sensitivity (proportion of true positives correctly identified by testing) and specificity (proportion of true negatives correctly identified by testing) values] obtained from the receiver operating characteristic (ROC) analysis [27]. The positive predictive value [the chance of disease given a positive result; number of cases true positive by testing/(number of cases true positive by testing + number of cases false positive by testing)], the negative predictive value [the chance of no disease given a negative result; number of cases true negative by testing/(number of cases true negative by testing + number of cases false negative by testing)], the likelihood ratio for positive result [the likelihood of having the disease, as opposed to not having the disease, having tested positive for it; sensitivity/(1 − specificity)] and sensitivity, specificity for earlier predefined cut-off points were calculated as generally recommended [28]. The total areas under the ROC curve (AUC) were measured to represent the probability of the tests correctly identifying true positives and negatives. The AUC of different tests [LNSC (ECLIA) measured once, the mean from two LNSC measurements (ECLIA), LNSC (ELISA), and DST] were directly compared on the ROC curve [29, 30].

SPSS 16.0 and Med Calc MedCalc(C) Version 10.4.6.0 software were used for the analysis.

Results

Demographic and anthropometric characteristics for all groups are summarized in Table 1.

The following reference range was calculated from the 98 LNSC samples of healthy volunteers: 0.5–9.4 nmol/l. The lowest detected LNSC was 0.5 nmol/l, and the maximum was 14.5 nmol/l. The 2.5–97.5 percentile calculated from healthy volunteers and obese patients ($n = 176$) was 0.5–12.5 nmol/l.

The results of two consecutive cortisol measurements for two separate days were available for 205 subjects and

Table 1 General characteristics of participants

	Cushing's syndrome	Constitutional obesity	Healthy volunteers
N (number)	45	78	98
Sex F:M (%)	35 (78 %):10 (22 %)	54 (69 %):24 (31 %)	73 (74 %):25 (26 %)
Age (years)	39.2 ± 13.3	37.5 ± 13.7	28.7 ± 7.5[b]
BMI (kg/m^2)	32.5 ± 6.7[a]	38.5 ± 6.6	22.5 ± 3.2[b]
Waist circumference	108.6 ± 14.9	115.2 ± 17.5	75.4 ± 11.2[b]
Hip circumference	107.0 ± 15.0[a]	124.4 ± 15.4	96.7 ± 7.2[b]

[a] Cushing's syndrome differs from constitutional obesity ($p < 0.001$)

[b] Healthy volunteers were younger, with lower BMIs and waist and hip circumferences ($p < 0.001$) than either Cushing's syndrome or constitutional obesity participants

Table 2 The results of two consecutive salivary cortisol measurements by ECLIA (Roche Cobas e601)

	Number of patients (*N*)	Late-night salivary cortisol level (nmol/l) median (Q25-Q75)		*p*
		First measurement	Second measurement	
Healthy volunteers	91	2.6 (1.6–4.1)	2.3 (1.5–4.0)	0.86
Constitutional obesity	72	3.2 (2.1–5.9)[a]	4.1 (2.1–6.9)[a]	0.29
Cushing's syndrome	42	21.9 (15.1–34.9)[b]	29.1 (8.8–45.4)[b]	0.14

[a] Patients with constitutional obesity had significantly higher salivary cortisol levels versus healthy volunteers ($p < 0.01$)

[b] Patients with Cushing's syndrome had significantly higher salivary cortisol levels versus both healthy volunteers and patients with constitutional obesity ($p < 0.001$)

are provided in Table 2. Reproducibility was assessed by looking at the day-to-day variabilities of patients and reflected by an interclass correlation coefficient of 0.785. ($p < 0.0001$).

LNSC levels in patients with constitutional obesity was significantly higher than in normal subjects ($p < 0.001$), which may be explained by the presence of functional hypercortisolism, a well-established condition in obese patients [7, 8]. A significant correlation was identified between BMI and LNSC in the pooled data of obese and healthy volunteers: the *R* (Spearman) was 0.26 ($p = 0.004$).

The ROC curve analysis of LNSC for CS patients and healthy volunteers had an AUC of 0.979 (95% CI 0.960–0.999). Maximum sensitivity and specificity was obtained for a cut-off value of 6.85 nmol/l. However, when CS patients and obese patients were analyzed together, the ROC curve analysis revealed an AUC of 0.953 (95% CI 0.918–0.987), with an optimal cut-off value of 9.4 nmol/l. The AUC (healthy volunteers and referred population vs. CS) was 0.968 (95% CI 0.943–0.992).

The diagnostic indices for the different thresholds are summarized in Table 3.

Maximal sensitivity of 100 % was observed for an LNSC level of 3.85 nmol/l. However, specificity with this threshold was unacceptably low at 59.0 % for patients with obesity, 72.5 %, for healthy volunteers, and 66.5 % for all participants.

Using predefined cut-off values, sensitivity and specificity of LNSC in our study was as follows: the threshold of 4.55 nmol/l [31] had sensitivity of 95.5 % and specificity of 69.0 % in obese patients versus 79.6 % in healthy subjects. A threshold of 8.3 nmol/l [32] had sensitivity of 84.4 % and specificity of 89.8 % in obese subjects compared to 96 % specificity in healthy volunteers.

Maximal specificity (100 %) was identified at cortisol levels of 19.7 nmol/l (sensitivity 62.2 %) in referred population. In healthy volunteers, a 14.5 nmol/l cut-off value had 100 % specificity and sensitivity of 80 %.

The absolute number of false positive and false negative results of LNSC during the first and second measurements (ECLIA) with cut-off value of 9.4 nmol/l and the percent of discordant results are summarized in Table 4. AUCs for the first [0.965 (95% CI 0.938–0.991)) and second (0.945 (95% CI 0.909–0.981)] LNSC measurements did not differ.

We suggested calculating the mean of two LNSC measurements. The AUCs for the means of two LNSC measurements from two consecutive days were 0.962 (95% CI 0.930–0.993) in the referred population and 0.986 (95% CI 0.972–1.000) in the healthy volunteers. The optimal cut-off value was determined to be 8.1 nmol/l for volunteers, with sensitivity of 91.1 % (95% CI 79.3–96.5) and specificity of 98 % (95% CI 92.8–99.4 %); the optimal cut-off for referred patients was 9.1 nmol/l with sensitivity of 88.9 % (95% CI 76.5–95.2) and specificity of 92.3 % (95% CI 84.2–96.4 %). Nevertheless, we did not identify statistically significant differences ($p = 0.447$) when comparing AUCs of single LNSC and the mean from two LNSC measurements.

Table 3 Diagnostic performance of late-night salivary cortisol measurements by ECLIA (Roche Cobas e601)

	Cushing's syndrome versus			
	Healthy volunteers	Constitutional obesity	Healthy volunteers	Constitutional obesity
Cut-off value	9.4 nmol/l		6.85 nmol/l	
Sensitivity (95% CI)	84.4 % (71.2–92.2)		91.1 % (79.3–96.5)	
Specificity (95% CI)	97.9 % (92.9–99.4)	92.3 % (84.2–96.4)	96.9 % (91.4–98.9)	84.6 % (75.0–90.9)
Positive predictive value (95% CI)	41.3 (10.4–164.0)	11.0 (5.0–23.9)	29.8 (9.7–91.0)	5.9 (3.5–10.0)
Negative predictive value (95% CI)	0.16 (0.08–0.31)	0.17 (0.08–0.33)	0.09 (0.04–0.23)	0.1 (0.04–0.27)
Likelihood ratio for positive result (95% CI)	260.5 (51.8–1,311.1)	65.1 (20.4–207.6)	324.5 (69.5–1,515.7)	56.4 (17.0–186.6)

95% CI 95% confidence interval

Table 4 The number of false positive, false negative results, and the percent of discordant results during the first and second measurement of late-night salivary cortisol (ECLIA) with a cut-off value of 9.4 nmol/l

	Number of false-positive results (above 9.4 nmol/l)		Number of false-negative results (below 9.4 nmol/l)		Absolute number and (percent) of discordant results
	LNSC (1)	LNSC (2)	LNSC (1)	LNSC (2)	
Cushing's syndrome			7	9	5 (11.9 %)
Constitutional obesity	6	7			7 (9.7 %)
Healthy volunteers	2	4			6 (6.6 %)

A high correlation (Spearman $R = 0.609$, $p < 0.001$) was identified between LNSC measured by ECLIA and ELISA in all participants. Hence, diagnostic performance of ELISA was very similar to ECLIA, with AUCs of 0.964 (95% CI 0.936–0.993) for referred patients and 0.972 (95% CI 0.949–0.994) for healthy volunteers. No significant differences were found between AUCs of LNSC measured by ECLIA and ELISA ($p = 0.375$). The predefined cut-off value (4 nmol/l) for LNSC had an unacceptably low specificity for both ELISA and ECLIA. LNSC measured by ELISA with a cut-off value of 9.4 nmol/l for obese patients yielded sensitivity of 88.4 % (95% CI 75.5–94.9 %) and specificity of 92.7 % (95% CI 84.5–96.9 %).

DST was performed in 120 patients from the referred population (three CS patients with severe conditions were omitted).

All patients with CS had DST above 50 nmol/l. In eight cases of constitutional obesity, dexamethasone failed to reduce cortisol below 50 nmol/l. Consequently, the cut-off value of 50 nmol/l [9] exhibited sensitivity of 100 % (95% CI 91.8–100 %) and specificity of 89.7 % (95% CI 78.5–94.4 %).

Among the eight patients whose cortisol levels were refractory to dexamethasone, six had LNSC values below 6.85 nmol/l, including four patients with LNSC below 4 nmol/l. In only two cases was the LNSC value above 9.4 nmol/l. Both initial tests (LNSC and DST) improved specificity for concordant results up to 97.4% (95% CI 91.1–99.3).

ROC curve analysis for DST showed an AUC = 0.982 (95% CI 0.959–1.00). No differences were identified between LNSC AUCs by ECLIA and DST ($p = 0.316$).

ROC curves and AUCs for all methods are presented in Fig. 1.

Discussion

This study demonstrated that LNSC measured by automated ECLIA has good diagnostic performance, comparable with earlier validated ELISA and DST, in the most challenging conditions, differentiating CS among consecutive obese and overweight patients. We also focused on the clinical implementation of two consecutive LNSC measurements and evaluated the order of testing to further improve diagnostic performance.

In suggesting the optimum cut-off value for LNSC based on the maximum sum of specificity and sensitivity, we noticed that the cut-off value was higher among the referral population (CS vs. obesity) as compared to CS versus healthy individuals, providing one possible explanation for the inconsistency of cut-off points in other studies. We suggest that differences in cut-off values actually depend on the number of patients who have functional hypercortisolism (due to obesity) compared to

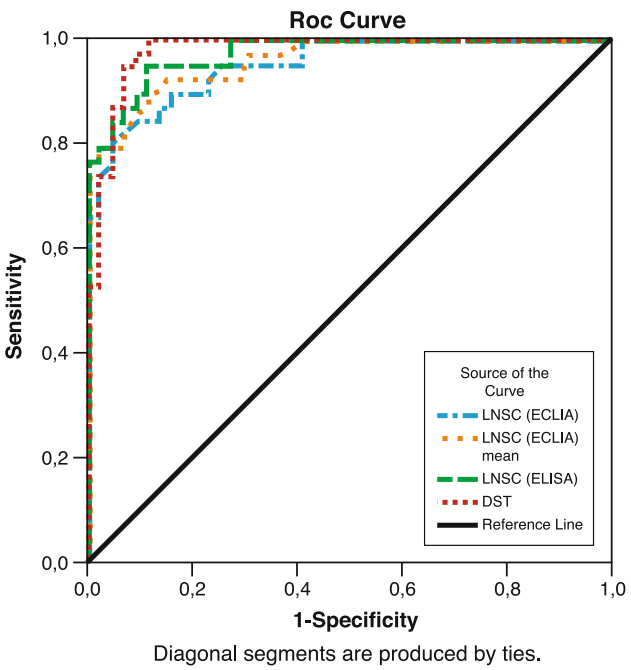

Fig. 1 The direct comparison of areas under the curve (AUC) of different tests to evaluate Cushing's syndrome in referred population of obese and overweight patients Receiver-operator characteristic curves of late-night salivary cortisol (LNSC) levels measured by the automated electrochemiluminescence immunoassay method (ECLIA); the mean of two LNSC measurements by ECLIA, LNSC measured by enzyme-linked immunosorbent assay (ELISA) and serum cortisol after low-dose dexamethasone suppression test (DST)

patients with incidentally discovered adrenal tumors or hypertension or healthy subjects who tend to lower the cut-off value.

Indeed, five studies [23, 31–34] focused on the evaluation of the ECLIA method revealed excellent diagnostic performance of the assay, but suggested cut-off points differed by almost 300 % ranging from 4.55 nmol/l [31] to 14.2 nmol/l [33]. Only one of these studies focused on patients with obesity versus CS in a case–control setting and reported the highest cut-off point (14.2 nmol/l) [33], whereas the study comparing healthy volunteers and patients with CS reported the lowest cut-off point [31]. The strength of our study is a prospective design regarding the evaluation of consecutive obese and overweight patients suspected of having CS. This approach is very close to real clinical practice, and therefore gives more accurate results.

Although our study focused on evaluating functional hypercortisolism versus CS, and salivary cortisol was measured using Cobas e 601, not Elecsys, the cut-off value obtained by our study of consecutive patients (9.4 nmol/l) was close to cut-offs earlier defined in Hungary from a prospective work −9.6 nmol/l (sensitivity—100 %; specificity—88 %) [23], 9.9 nmol/l (sensitivity—91.3 %; specificity—94.5 %) [34]. It clearly shows the potential for this method being standardized. The exclusion of four patients with an unproved diagnosis from the analysis could be considered as the limitation of our study.

However, the largest number of patients with CS at all stages of disease tested with the ECLIA method to date (45 vs. 9 [23] or 23[34]) let us more accurately analyze diagnostic performance of testing, particularly sensitivity, which was 84 %. We also provided the cut-off point with 100 % sensitivity; with 100 % specificity, and diagnostic indexes for earlier predefined cut-off values in case they were required for future analysis.

Our healthy volunteer data, obtained using the latest Cobas e601, agrees with previous studies analyzing the reference range of LNSC collected at 23:00 as assayed by ECLIA on Elecsys 170 [31, 35].

The principal interests of our study were the analysis of clinical implementation of two consecutive LNSC samplings, comparison of diagnostic performance of two methods to measure LNSC (ECLIA vs. ELISA), and evaluation of the optimal combination of diagnostic tests.

Comparing AUCs from the first, second, and the mean from the two LNSC measurements reveals similar diagnostic performance. The number of false-positive and false-negative results during the first and second measurements and percent of discordant results was similar in all groups. Carrasco et.al. [36] evaluated the results of two LNSC measurements in 26 patients with CS and 35 subjects from a clinically suspected group (all had normal 24 h UFC and DST) and suggested that the highest LNSC should be chosen to improve diagnostic performance. In our study of consecutive 123 obese and overweight patients, such an approach resulted in an unacceptably low specificity, and thus could not be recommended. Variations in LNSC results could arise from stressful conditions. Nevertheless, it is difficult to predict which day or what conditions might be stressful for individuals. Consequently, recommending a second sample for every patient would be equally misleading.

Diagnostic performance of LNSC measured by ECLIA and ELISA did not differ. Moreover, the cut-off point 9.4 nmol/l yielded similar sensitivity and specificity. Consequently, we can substitute previously validated ELISA [9] with ECLIA. Automated ECLIA techniques allow results to be easily determined on the same day that samples are delivered. This is an improvement over ELISA, where it is necessary to collect and freeze a specific number of samples to optimize kit capacity.

Thus, our study does not support two LNSC measured by either one method or two different methods (ECLIA and than ELISA). Instead, we advocate the initial use of LNSC measured by ECLIA followed by DST to improve sensitivity and specificity for concordant results. This approach gave false positive in only two cases of constitutional obesity and no false negative result.

In conclusion, ECLIA-based LNSC measurements are comparable to those from ELISA or DST. Different thresholds should be used for patients with functional hypercortisolism, such as obesity. Based on our results, two consecutive LNSC measurements should not be mandatory; rather, DST should be used as a second test to confirm or exclude CS.

Acknowledgments This work was supported by the Presidential Grant no. MK-6978.201

Disclosure The authors have nothing to disclose.

References:

1. C. Steffensen, A.M. Bak, K.Z. Rubeck, J.O.L. Jorgensen, Epidemiology of Cushing's syndrome. Neuroendocrinology **92**(1), 1–5 (2010)
2. B. Catargi, V. Rigalleau, A. Poussin, N. Ronci-Chaix, V. Bex, V. Vergnot, H. Gin, P. Roger, A. Tabarin, Occult Cushing's syndrome in type-2 diabetes. J. Clin. Endocrinol. Metab. **88**, 5808–5813 (2003)
3. G. Leibowitz, A. Tsur, S.D. Chayen, M. Salameh, I. Raz, E. Cerasi, D.J. Gross, Pre-clinical Cushing's syndrome an unexpected frequent cause of poor glycemic control in obese diabetic patients. Clin. Endocrinol. (Oxf). **44**, 712–722 (1996)
4. M. Omura, K. Yamaguchi, Y. Kakuta, T. Nishikawa, Prospective study on the prevalence of secondary hypertension among hypertensive patients visiting a general outpatient clinic in Japan. Hypertens. Res. **27**, 193–202 (2004)

5. I. Chiodini, M.L. Mascia, S. Muscarella, C. Battista, S. Minisola, M. Arosio, S.A. Santini, G. Guglielmi, V. Garnevale, A. Scillitani, Subclinical hypercortisolism among outpatients referred for osteoporosis. Ann. Intern. Med. **147**, 541–548 (2007)
6. I. Chiodini, M. Torlontano, A. Scillitani, M. Arosio, S. Bacci, S. Di Lembo, P. Epaminonda, G. Augello, R. Enrini, B. Ambrosi, G. Adda, V. Trischitta, Association of subclinical hypercortisolism with type 2 diabetes mellitus: a case–control study in hospitalized patients. Eur. J. Endocrinol. **153**, 837–844 (2005)
7. C. Davenport, A. Liew, B. Doherty, H.H.N. Win, H. Misran, S. Hanna, D. Kealy, F. Al-Nooh, A. Agha, C.J. Thompson, M. Lee, D. Smith, The prevalence of adrenal incidentaloma in routine clinical practice. Endocrine **40**, 80–83 (2011)
8. D.A. Vassiliadi, G. Ntali, T. Stratigou, M. Adali, S. Tsagarakis, Abberant cortisol responses to physiological stimuli in patients presenting with bilateral adrenal incidentalomas. Endocrine **40**, 437–444 (2011)
9. L.K. Nieman, B.M.K. Biller, J.W. Finding, J. Newell-Price, M.O. Savage, P.M. Stewart, V.M. Montori, The diagnosis of Cushing's syndrome: an endocrine society clinical practice guideline. J. Clin Endocrinol Metab. **93**, 1526–1540 (2008)
10. P. Putignano, P. Toja, A. Dubini, P.F. Giraldi, S.M. Corsello, F. Cavaqnini, Midnight salivary cortisol versus urinary free and midnight serum cortisol as screening tests for Cushing's syndrome. J. Clin. Endocrinol Metab **88**, 4153–4157 (2003)
11. J.P. Kahn, D.R. Rubinov, C.L. Davis, M. Kling, R.M. Post, Salivary cortisol: a practical method for evaluation of adrenal function. Biol. Psychiatry **23**, 335–349 (1988)
12. J. Guechot, J. Fiet, P. Passa, J.M. Villette, B. Gourmel, F. Tabuteau, G. Cathelineau, Dreux C Physiological and pathological variations in saliva cortisol. Horm. Res. **16**, 357–364 (1982)
13. P.J. Evans, J.R. Peters, J. Dyas, R.F. Walker, D. Riad-Fahmy, R. Hall, Salivary cortisol levels in true and apparent hypercortisolism. Clin Endocrinol. (Oxf). **20**, 709–715 (1984)
14. H. Raff, J.L. Raff, J.W. Finding, Late-night salivary cortisol as a screening test for Cushing's syndrome. J. Clin. Endocrinol. Metab. **83**, 2681–2686 (1998)
15. D.A. Papanicolaou, N. Mullen, I. Kyrou, L.K. Nieman, Nighttime salivary cortisol: a useful test for the diagnosis of Cushing's syndrome. J. Clin. Endocrinol. Metab. **87**, 4515–4521 (2002)
16. A. Viardot, P. Huber, J.J. Puder, H. Zulewski, U. Keller, B. Muller, Reproducibility of nighttime salivary cortisol and its use in the diagnosis of hypercortisolism compared with urinary free and overnight dexamethasone suppression test. J. Clin. Endocrinol. Metab. **90**, 5730–5736 (2005)
17. S. Kidambi, H. Raff, J.W. Finding, Limitation of nocturnal salivary cortisol and urine free cortisol in the diagnosis of mild Cushing's syndrome. Eur. J. Endocrinol. **157**, 725–731 (2007)
18. T. Deutschbein, N. Unger, J. Hinrichs, M.K. Walz, K. Mann, S. Petersen, Late-night and low-dose dexamethasone-suppressed cortisol in saliva and serum for the diagnosis of cortisol-secreting adrenal adenoma. Eur. J. Endocrinol. **161**, 747–753 (2009)
19. S. Sakihara, K. Kageyama, Y. Oki, M. Doi, Y. Iwasaki, S. Takayasu, T. Moriyama, K. Terui, T. Nigawara, Y. Hirata, K. Hashimoto, T. Suda, Evaluation of plasma, salivary, and urinary cortisol levels for diagnosis of Cushing's syndrome. Endocr. J. **57**, 331–337 (2010)
20. H. Raff, S.L. Ettema, D.C. Eastwood, B.T. Woodson, Salivary cortisol in obstructive sleep apnea: the effect of CPAP. Endocrine **40**, 137–139 (2011)
21. K.I. Alexandraki, A.B. Grossman, Novel insights in the diagnosis of Cushing's syndrome. Neuroendocrinology **92**(1), 35–43 (2010)
22. S.K. Baid, N. Sinaii, M. Wade, D. Rubino, L.K. Nieman, Radioimmunoassay and tandem mass spectrometry measurement of bedtime salivary cortisol levels: a comparison of assays to establish hypercortisolism. J. Clin. Endocrinol. Metab. **92**, 3102–3107 (2007)
23. G. Beko, I. Varga, E. Glaz, M. Sereg, K. Feldman, M. Toth, K. Racz, A. Patocs, Cutoff values of midnight salivary cortisol for the diagnosis of overt hypercortisolism are highly influenced by methods. Clin. Chim. Acta **411**, 364–367 (2010)
24. S.M. Baid, D. Rubino, N. Sinaii, S. Ramsey, A. Frank, L.K. Nieman, Specificity of screening tests for Cushing's syndrome in an overweight and obese population. J. Clin. Endocrinol. Metab. **94**, 3857–3864 (2009)
25. M. Boscaro, G. Arnaldi, Approach to the patient with possible Cushing's syndrome. J. Clin. Endocrinol. Metab. **94**, 3121–3131 (2009)
26. M. Nunes, S. Vattaut, J. Corcuff, A. Rault, H. Loiseau, B. Gatta, N. Valli, L. Letenneur, A. Tabarin, Late-night salivary cortisol for diagnosis of overt and subclinical Cushing's syndrome in hospitalized and ambulatory patients. J. Clin. Endocrinol. Metab. **94**, 456–462 (2009)
27. K.H. Zou, J. O'Maley, L. Mauri, Receiver-operating characteristic analysis for evaluating diagnostic tests and predictive models. Circulation **115**, 654–657 (2007)
28. O Ajetunmobi, Making sense of critical appraisal (Hodder Arnold part of Hachette, London, 2002). pp. 69–84
29. J.A. Hanley, B.J. McNeil, The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology **143**, 29–36 (1982)
30. J.A. Hanley, B.J. McNeil, A method of comparing the areas under receiver operating characteristic curves derived from the same cases. Radiology **148**, 839–843 (1983)
31. K. Jeyaraman, A.C. Amini, G. Nandita, S.N. Dwivedi, Late-night salivary cortisol in normal subjects and in patients with Cushing's syndrome. Postgrad Med J. **86**, 399–404 (2010)
32. C. Carrozza, S.M. Corsello, R.M. Paragliola, F.I.S. Palumbo, P. Locantore, A. Sferrazza, A. Pontecorvi, C. Zuppi, Clinical accuracy of midnight salivary cortisol measured by automated electrochemiluminescence immunoassay method in Cushing's syndrome. Ann. Clin. Biochem. **47**, 228–232 (2010)
33. M. Yaneva, G. Kirilov, S. Zacharieva, Midnight salivary cortisol, measured by highly sensitive electrochemiluminescene immunoassay, for the diagnosis of Cushing's syndrome. Cent. Eur. J. Med. **4**, 59–64 (2009)
34. M. Sereg, J. Toke, A. Patocs, I. Varga, P. Igaz, N. Szucs, J Horanyi, P. Pusztai, S. Czirjak, E. Glaz, K. Racz, M. Toth, Diagnostic performance of salivary cortisol and serum osteocalcin measurements in patients with overt and subclinical Cushing's syndrome. Steroids. **76**, 38–42 (2011)
35. M. Vogeser, J. Durner, E. Seliger, C. Auernhammer, Measurement of late-night salivary cortisol with an automated immunoassay system. Clin. Chem. Lab. Med. **44**, 1441–1445 (2006)
36. CA Carrasco, M Garcia, M Goycoolea, J Cerda, J Berherat, O. Padilla, D. Meza, N. Wohllk, T Quiroga, Reproducibility and performance of one or two samples of salivary cortisol in the diagnosis of Cushing's syndrome using an automated immunoassay system. Endocrine. (2012). doi:10.1007/s12020-012-9597-z